(54) Title: GLYCOSYLATED LEPTIN COMPOSITIONS AND RELATED METHODS

(57) Abstract

The present invention relates to glycosylated leptin compositions and related methods. Included are glycosylated leptin proteins having a Stokes' radius allowing for improved properties, as well as glycosylated leptin proteins having selected sites for glycosylation, nucleic acids encoding such proteins, related host cells, vectors, processes for production, and methods of use of such compositions. Novel methods of producing glycosylated proteins are also provided. The glycolysated leptin protein can be used for preparing a pharmaceutical composition that can be used in the treatment of a human for a condition selected among obesity, diabetes and high blood lipid content.

GLYCOSYLATED LEPTIN COMPOSITIONS AND RELATED METHODS

Field of the Invention

The present invention relates to glycosylated
5   leptin compositions and related methods. Included are
glycosylated leptin proteins having a Stokes' radius
allowing for improved properties, as well as
glycosylated leptin proteins having selected sites for
glycosylation, nucleic acids encoding such proteins,
10   related host cells, vectors, processes for production,
and methods of use of such compositions. Novel methods
of producing glycosylated proteins are also provided.

Background

Although the molecular basis for obesity is
15   largely unknown, the identification of the "OB gene"
and protein encoded ("OB protein," also referred to
herein as "leptin") has shed some light on mechanisms
the body uses to regulate body fat deposition. Zhang
et al., Nature 372: 425-432 (1994) incorporated herein
20   by reference; see also, the Correction at Nature 374:
479 (1995) also incorporated herein by reference. The
OB protein is active in vivo in both ob/ob mutant mice
(mice obese due to a defect in the production of the OB
gene product) as well as in normal, wild type mice.
25   The biological activity manifests itself in, among
other things, weight loss. See generally, Barinaga,
"Obese" Protein Slims Mice, Science 269: 475-476
(1995). See PCT International Publication Number
WO96/05309, "Modulators of Body Weight, Corresponding
30   Nucleic Acids and Proteins, and Diagnostic and
Therapeutic Uses Thereof," herein incorporated by
reference in its entirety. See also, PCT International
Publication Numbers WO96/40912, WO97/06816, WO97/18833,

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WO97/38014, WO98/08512, and WO98/28427, all of which
describe OB methods and compositions in greater detail
and are all herein incorporated by reference in their
entirety.

5   The other biological effects of OB protein
are not well characterized. See generally, Friedman et
al., Nature 395: 763-770 (October 1998) for a review of
leptin and the regulation of body weight in mammals,
herein incorporated by reference. It is known, for

10   instance, that in ob/ob mutant mice, administration of
OB protein results in a decrease in serum insulin
levels, and serum glucose levels. It is also known
that administration of OB protein results in a decrease
in body fat. This was observed in both ob/ob mutant

15   mice, as well as non-obese normal mice. Pelleymounter
et al., Science 269: 540-543 (1995); Halaas et al.,
Science 269: 543-546 (1995). See also, Campfield et
al., Science 269: 546-549 (1995) (Peripheral and
central administration of microgram doses of OB protein

20   reduced food intake and body weight of ob/ob and
diet-induced obese mice but not in db/db obese mice.)
In none of these reports have toxicities been observed,
even at the highest doses.

          Recombinant leptin is effective in humans to

25   result in weight loss. Greenberg AS, Heymsfield SB,
Fujioka K, et al., Preliminary safety and efficacy of
recombinant methionyl human leptin (rL) administered by
SC injection in lean and obese subjects. Poster
presented at: 58th Annual Meeting and Scientific

30   Sessions of the American Diabetes Association; June 14,
1998; Chicago, IL, herein incorporated by reference.
As has been demonstrated, administration of recombinant
methionyl human leptin to obese humans has resulted in

weight loss without toxicities. Further, the weight
that is lost is predominantly fat. Heymsfield et al.,
Weight and body composition changes in lean and obese
subjects treated with recombinant methionyl human
5   leptin. Poster presented at: International Congress on
Obesity; August 29 - September 3, 1998; Paris, France,
herein incorporated by reference.

Native human leptin is known to have a
relatively fast half life in humans. Lau et al.,
10   Pharmacokinetics of recombinant methionyl human leptin
and the effect of antibody formation in lean and obese
subjects following subcutaneous dosing. Poster
presented at: International Congress on Obesity; August
29-September 3, 1998, Paris, France, herein
15   incorporated by reference. In the systemic
circulation, accumulation may be accomplished either by
giving larger doses or more frequent doses of the
subject protein. Reports indicate that exogenous, as
well as endogenous leptin is removed from the
20   circulation, at least in part, by the kidney. See,
e.g., Cumin et al., Journal of Endocrinology, 155: 577-
585 (1997) and Cumin et al., Internal Journal of
Obesity 21: 495-504 (1997), both herein incorporated by
reference.

25   In general, the kidney functions to clear the
blood plasma of certain substances by concentrating
them in the urine. See, e.g., Harth, The Function of
the Kidneys, In: Human Physiology, Schmidt et al.,
eds., Springer-Verlag New York, Heidelberg, Berlin,
30   1983 at pp. 610-642, herein incorporated by reference.
The rate or degree to which a serum protein may pass
through the kidney is difficult to estimate, but, in
general, the kidney anatomy allows for free passage of

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water and small solutes, but imposes a barrier to the
passage of plasma proteins. Different substances have
different "filterability", kidney clearance rates, see,
Anderson et al., Renal and Systemic Manifestations of
5   Glomerular Disease, In: The Kidney, Brener et al.,
eds., Harcort Brace Joanovich, Inc., Philadelphia, PA
1991 at pages 1831-1843, herein incorporated by
reference.

Leptin may be accumulated in the systemic
10   circulation by continuous administration, such as by
osmotic pump or by chemically derivatizing the protein
so that the circulation time is increased. See, e.g.,
PCT WO96/40912, published December 19, 1996 and herein
incorporated by reference in its entirety. Chemical
15   derivatization of a recombinantly-produced protein
generally requires, however, a two (or more) step
process: step one, make the protein; step two, add a
chemical moiety (such as a polyethylene glycol or
dextran moiety), see, e.g., PCT WO96/40912, supra, at
20   page 8 et seq. for a description of N-terminally
derivatizing leptin (therein referred to as OB
Protein).

For a "one step" process, in a recombinant
DNA system, one may encode a fusion protein
25   (alternatively called a "chimeric" protein) where an
additional polypeptide moiety is encoded along with the
desired protein, so that both are expressed.
Lengthening the protein may also increase circulation
time. Polypeptides such as the "Fc" portion of an
30   antibody, or albumin have been used in this regard.
See e.g., PCT WO 98/28427, herein incorporated by
reference, entitled, "OB Fusion Protein Compositions
and Methods." The general disadvantage in

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manufacturing is that larger expression products are
sometimes more difficult to fold into proper
conformations, and yields may be lower than those for
smaller products. Further, the overall protein load per
5   dose is increased, and the proportion of therapeutic
protein is decreased, with the use of increasingly
large sized fusion proteins.

            The presence of carbohydrate on a protein can
affect its clearance rate and may improve its potency
10   in vivo, while at the same time it can affect the
protein's intrinsic activity, solubility, stability and
immunogenicity. See, e.g. , European Patent
Publication 0 640 619, published March 1, 1995,
entitled, "Erythropoietin analogs with additional
15   glycosylation sites," herein incorporated by reference,
and PCT patent publication WO 96/25498, published
August 22, 1996, entitled, "MPL Ligand Analogs" both
herein incorporated by reference.

            Furthermore, carbohydrate may be added by
20   eukaryotic cell production, without the need for a
two-step process. E.g., PCT/US96/06609, published
November 14, 1996, herein incorporated by reference,
proposes various mammalian signal sequences for
secretion of an ob protein from a mammalian cell (at
25   pages 11-12, for example). See also, PCT WO 97/20933,
published June 12, 1997, entitled, "Mutational Variants
of Mammalian OB Gene Proteins," particularly at page
11, which proposes OB protein glycosylation
alterations. Glycosylation occurs at specific locations
30   along the polypeptide backbone. There are usually two
types of glycosylation: O-linked oligosaccharides are
attached to serine or threonine residues while N-linked
oligosaccharides are attached to asparagine residues

when they are a part of the sequence Asn·X·Ser/Thr, where X can be any amino acid except proline. The structures of N·linked and O·linked oligosaccharides and the sugar residues found in each type are

5   different. One type of sugar that is commonly found on both is N·acetylneuraminic acid (hereafter referred to as sialic acid). Sialic acid is usually the terminal residue of both N·linked and O·linked oligosaccharides in mammalian cells and, by virtue of its negative

10   charge, may confer acidic properties to the glycoprotein. The predominate form of naturally occurring human leptin (provided in human cells) is not glycosylated. A variant of the naturally occurring protein having a glutamine absent at position 28 of the

15   mature protein (SEQ ID NO. 2, _infra_) does contain two sites for glycosylation. These sites are both for O·linked glycosylation. It is believed, however, that this form is produced in only trace amounts in humans, and is not the predominant active form _in vivo_.

20   It would be desirable to have a process, which results in a leptin having an increased systemic circulation time, which does not require such second derivatization step as described above. It is further desirable to increase the intrinsic activity, and

25   solubility of leptin, without causing or increasing immunogenicity or other detrimental effects.

Summary of the Invention

         The present invention stems from the
30   observation that, as compared to unaltered native recombinant human leptin, glycosylated leptin protein is functional _in vivo_, and, further, certain forms of

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glycosylated leptin protein have longer systemic
circulation times in vivo, without toxicities.

It has been found, surprisingly and
importantly, that a glycosylated human leptin having a
5   single N-linked glycosylation site has in vitro as well
as in vivo biological activity. Further, the
biological activity is equal to or is slightly more
potent than recombinant human native leptin protein. As
indicated above, leptin's effect on obesity is thought
10   to be due, in part, by action in the brain. As
indicated above, leptin is not a naturally glycosylated
molecule (in the Q+28 form, SEQ ID NO. 1, infra, which
is believed to be the predominant form in human serum).
Further, glycosylated proteins (glycoproteins)
15   generally may not enter the brain because of an
inability to cross the blood-brain barrier.
Demonstration of equal (or slightly better) biological
activity by glycosylated leptin demonstrates either
that (a) the glycosylated leptin enters the brain, or
20   (b) if it doesn't, glycosylated leptin is more
biologically effective in the peripheral tissues (such
as visceral areas of adipose tissue) than native
recombinant human leptin.

It has further been observed that a human
25   leptin which is N-glycosylated at three sites has a far
longer circulation time and potency than recombinant
native human leptin or leptin N-glycosylated at a
single site. As set forth in the working examples
below, various two-, three-, four-, and five- site
30   glycosylated leptins have been prepared and tested for
in vitro, and in some cases, in vivo activity.

The present glycosylated leptins may have a
desired, relatively long plasma half life. The present

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glycosylated leptins which have Stokes' radius of greater than or equal to 30Å have a reduced rate of filterability through the membranes, and thus a reduced rate of degradation in the kidney.

5   Although Stokes' radii may be determined in a variety of ways, the preferred way herein is to use gel filtration chromatography. See generally, Le Maire et al., Analytical Biochemistry 154: 525-535 (1986) herein incorporated by reference, for gel filtration

10   chromatography for determining the Stokes' radii of various proteins for use as standards. Thus, the present glycosylated leptins are those which have a Stokes' radii of about 30 Å when determined using gel filtration chromatography.

15   It is preferred that the present glycosylated leptins also substantially avoid clearance in the liver. The liver is known to have receptors which bind galactose. Galactose is a sugar and may be a component of the carbohydrate moiety on the present glycosylated

20   leptins. Sialic acid will typically "cap" the galactose moiety, and prevent its reactivity with galactose receptors in the liver. Additionally, a sialic acid moiety imparts a negative charge. The more negatively charged the present glycosylated leptins

25   are, the more they will "repel" the negatively charged membranes of the liver and kidney. Therefore, the present glycosylated leptin proteins are preferably those having at least a majority of the galactose moieties unavailable for binding to a galactose

30   receptor, and more preferably, having a sialic acid moiety located at least a majority of the sites available for sialation.

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As discussed herein, the recombinant human
leptin modified to contain sites for N-linked
glycosylation at one site or at three sites,
demonstrated that glycosylated leptin protein could be
5   functional, and could be as functional as natural
human. Glycosylation was accomplished via host cellular
machinery, in cell culture, and therefore did not
require an extra process step (as required to
derivatize protein) to attain the desired
10 characteristics of longer serum half life.

Thus, in one aspect, the present invention
relates to a glycosylated leptin protein having a
Stokes' radius greater than that of naturally occurring
glycosylated human leptin of SEQ ID NO. 2 (rHu-Leptin
15 1-145, below). In another aspect, the present invention
relates to a glycosylated leptin protein having a
Stokes' radius greater than that of a glycosylated
leptin protein having one N-linked glycosylation
moiety. And, in yet another aspect, the present
20 invention relates to a glycosylated leptin protein
having a Stokes' radius equal to or greater than 30 Å,
as determined by gel filtration.

The present invention also relates to leptin
proteins having at least one additional glycosylation
25 site. In yet another way, the present invention relates
to a glycosylated leptin protein having five or greater
than five sialic acid moieties. The naturally occurring
human leptin variant (SEQ. ID NO: 2, below) contains 2
sites for O-linked glycosylation, and therefore may
30 contain 4 sialic acid moieties. The present working
examples demonstrate that more heavily glycosylated
leptin protein has substantially improved circulation
time. Further, the present invention relates to a

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glycosylated leptin protein having five, six or seven
sialic acid moieties.

In other aspects, the present invention
relates to a nucleic acid encoding a glycosylated
5   leptin protein as set forth herein, as well as a vector
containing a nucleic acid encoding a glycosylated
leptin protein according to the disclosure herein.

Thus, in yet other aspects, the present
invention relates to a host cell containing a nucleic
10   acid encoding a glycosylated leptin protein according
to the present disclosure.

The present invention also relates to use of
the present nucleic acids for gene therapy.
Further, the present invention relates to a method of
15   preparing a glycosylated leptin protein.

The present invention also relates to
selective binding molecules, such as antibodies which
selectively bind the present glycosylated leptin
proteins.

20   In other aspects more fully described below,
the present invention relates to a pharmaceutical
compositions comprising a glycosylated leptin protein
of the present invention in a pharmaceutically
acceptable carrier. The present invention also relates
25   to a method of treatment of a human for a condition
selected from among obesity, diabetes, and high blood
lipid content effects; said method comprising
administering an effective amount of a glycosylated
human leptin according to the present invention.

30   The present invention also relates to
improved methods of production of glycosylated leptin
proteins, as well as production of glycosylated
proteins generally. The present working examples

demonstrate that for the present glycosylated leptin
proteins, use of a signal peptide other than the native
human leptin signal peptide improve glycosylation
efficiency. In this case, improved glycosylation
5   efficiency results in the desirable property of both
increased number and size of added carbohydrate chains.
Thus, the present compositions and methods include the
use of signal peptides other than the native leptin
signal peptide. Apart from the native human leptin
10 signal, particular signal peptides, both those known to
be naturally found (i.e., natural signal peptides are
those which have not been genetically manipulated by
humans, by any means including homologous
recombination, recombinant DNA techniques, or other
15 means known or expected to alter the nucleic acid
sequence constituents, although the cell containing
them may have been cultured or otherwise removed from
its natural in vivo environment), as well as those not
found in nature (i.e., non-natural signal peptides are
20 those which have been genetically manipulated by humans
as described above), are set forth below.

      The present invention further relates to the
observation that modification of signal peptides, as
well as other peptides which are processed off of the
25 mature protein, improve the yield of glycosylated
proteins. Signal peptide modifications include
alteration of the peptidase cleavage site to improve
cleavage accuracy (and thus produce a larger yield of
desired glycosylated proteins having the predicted N-
30 terminal amino acid sequence). Signal peptide
modifications may also, or alternatively, largely
improve glycosylation efficiency, even in the absence
of "correct" cleavage of the mature protein from the

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presequences. ("Correct" indicating that the first
amino acid on the N-terminus is one for the predicted
mature protein, not having any amino acids found on the
signal peptide or other presequences). Other
5   modifications include the addition of "prosequences"
which are also cleaved off but also generate improved
glycosylation efficiency. Natural as well as non-
naturally occurring signal peptides may be modified as
such. Specific examples are provided herein.

10   Therefore, the present invention also relates
to an improved method of manufacturing a glycosylated
protein comprising:

(a) culturing, under suitable conditions for
expression and glycosylation, a host cell containing a
15   DNA sequence encoding, in the 5' to 3' direction (i) a
signal peptide, and (ii) a DNA encoding a glycosylated
protein; and

(b) obtaining said glycosylated protein
wherein said improvement comprises use of a signal
20   peptide having a peptidase cleavage site optimized for
maximizing yield of said glycosylated protein, and,
optionally, the addition of a prosequence.

Brief Description of the Figures

25   FIGURE 1 is a graph showing weight loss relative to
buffer control for animals dosed with various doses of
a one-site glycosylated leptin ("Glycosylated CHO
Leptin) and non-glycosylated rmetHu-Leptin1-146
("Leptin").

30   FIGURE 2 is a Western Blot, as further described in
Examples 5 and 6, below, showing that alterations in

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amino acid sequence of the glycosylation site can alter the kind or amount of glycosylation.

FIGURE 3 is a graph of serum leptin concentrations following subcutaneous administration of 1.0 mg/kg
5   rmetHu-Leptin or a three-site glycosylated leptin protein in male CD-1 mice as further described in Example 7.

FIGURE 4 is a graph of serum leptin concentrations following intravenous administration of 1.0 mg/kg
10   rmetHu-Leptin or a three-site glycosylated leptin protein in male CD-1 mice as further described in Example 7.

FIGURE 5 is a graph of weight loss upon administration of a three-site glycosylated leptin protein ("GE-
15   Leptin") as further described in Example 8

FIGURE 6 is a graph of food intake upon administration of a three-site glycosylated leptin protein ("GE-Leptin") as further described in Example 9.

FIGURE 7 is a Western Blot illustrating the effects of
20   various signal peptides on expression and glycosylation of a three-site glycosylated leptin protein, as further described in Example 14.

FIGURE 8 is a Western Blot illustrating the effects of various signal peptides, and other peptides, on the
25   glycosylation of a three-site glycosylated leptin, as further described in Example 14.

FIGURE 9 is a Western Blot illustrating the effects of the peptidase cleavage site on glycosylation of a three-site glycosylated leptin protein as further
30   described in Example 14.

FIGURE 10 is a Western Blot illustrating the effects of various signal peptides and other peptides on the

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glycosylation of a three-site glycosylated leptin as further described in Example 14.

FIGURE 11 is a Western Blot, as described in Example 15 below, and shows that increasing the number of

5   glycosylation sites, at least up to five sites, increases the amount of glycosylation found on the leptin protein when expressed in CHO cells.

Detailed Description of the Invention

As indicated above, the present invention

10 relates, in one aspect, to glycosylated leptin proteins having a Stokes' radius larger than that of naturally occurring glycosylated human leptin. Preferably for increasing half life of a therapeutic composition in the systemic circulation of the Stoke's radius is of

15 sufficient size to reduce filterability in the kidney. The effect of having a Stokes' radius of this size is to keep the glycosylated leptin protein in the systemic circulation for a longer period of time than would be for a glycosylated, or other leptin protein, not having

20 this effective size. Upon empirical determination of the Stokes' radius for the present glycosylated leptin protein, the size must be greater than or equal to about 30 Å, as determined by methods described in further detail below. When used with reference to an

25 individual glycosylated leptin protein molecule, the term "about" means the average Stokes' radius over a period of time for that individual glycosylated leptin protein molecule.

As provided herein, glycosylated leptin

30 proteins having a Stoke's radius greater than naturally occurring leptin proteins has improved properties. The preferred Stokes' radius for a population of glycosylated leptin protein molecules, such as is

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present in a therapeutically effective dose, is that
which is greater than or equal to about 30 Å. The term
"about" here indicating that of any population of
glycosylated leptin protein molecules, some may have a
5   greater Stokes' radius, some may have a lower Stokes'
radius, but the mean Stokes' radius of a given
population of glycosylated leptin protein is greater
than or equal to 30 Å.

The higher above 30 Å Stokes' radius, the
10   larger the effective size of the glycosylated leptin
protein molecule(s). The larger the effective size,
i.e., the larger the hydrodynamic volume attained by
the addition of oligosaccharide, the slower the effect
movement through basement membranes throughout the
15   body. In order for leptin to reach the kidney tubules
where it is degraded it must first pass through the
basement membranes of the glomerulus. Thus, increasing
the hydrodynamic size slows filtration through the
glomerular membrane, and therefore slows degradation,
20   and thus ultimate clearance, of the polypeptide in the
proximal tubules. For example, the present 3-
glycosylation site leptin, rHu-Leptin 1-146 with
glycosylation sites at position 47, 69, and 102 (i.e.,
having an asparagine residue substituted at positions
25   47, 69 and 102, and a threonine residue substituted at
position 29, 71 and 104) has a mean Stokes' radius of
32.1 Å (based on two gel filtration measurements of
31.9 Å and 32.3 Å). The working example below
demonstrates that this glycosylated leptin exhibited a
30   4- to 5-fold decrease in systemic clearance and
increase in half-life compared to rmetHu-Leptin.

Also as indicated above, there are several
ways to determine the Stokes' radius of a molecule.

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The present Stokes' radius, for purposes of the present
glycosylated leptin proteins, is determined using gel
filtration, see, Le Maire et al., supra, see also,
Kyte, Structure in Protein Chemistry, Garland

5   Publishing, Inc., New York and London, 1995 at pages
293-316, herein incorporated by reference. Presently,
the gel filtration used to determine Stokes' radius is
polymer (agarose) beads to which dextran is covalently
bound. Commercial preparations include Superdex^{tm} 200

10   HR 10/30 (Pharmacia) and Sephacryl®S-200 high
Resolution (Pharmacia). These two preparations were
alternatively used to determine the Stokes' radius of
the present glycosylated leptin proteins.

A column may be of any size but a size of
15   approximately 1 x 30 cm is preferred for ease in
handling. The instruction manuals for column
preparation for each of the above gel filtration
substances are herein incorporated by reference in
their entirety (Paper number 71-7059-00 Edition AB for

20   Superdex ^{tm} and 52-2086-00-03, for Sephacryl®).

The buffer to be used should be fairly close
to a physiological buffer that does not significantly
alter the solution conformation of the molecule and
interfere with the size separation of the protein

25   molecules. Phosphate buffered saline is preferred, and
was used to determine the Stokes' radius of the present
glycosylated leptin proteins.

The process for performing gel filtration
should generally follow the instruction manuals as
30   above incorporated. The selected glycosylated leptin
protein for which a Stokes' radius is to be determined
should be loaded onto the column. Below, for example, a
concentration of 0.4 A280/ml, which is about 0.45

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mg/ml, was used for a three-site glycosylated leptin
(47,49,102). The buffers used herein was PBS, but
other buffers may be used. The buffer for the load
should be compatible with good gel filtration practices

5   and could in theory contain high salts, and other
materials consistent with what one skilled in the art
would consider appropriate. The load or storage buffer
should not interfere (either by precipitating as it
hits the column or by being denaturing and requiring a

10   refold as it elutes) with determination of the Stokes
radius. A column of gel filtration substances which has
not previously been used, is preferred. The washing
buffer, such as phosphate buffered saline, should be
applied at a rate of 0.25 ml/min or a linear flow rate

15   of 0.3 cm/min. This value will be determined by the
properties of the gel and is basically per the
manufacturers instructions. The fractions eluted
contain the glycosylated leptin protein molecules which
are not trapped in the gel filtration substance.

20   To determine the Stokes' radius, it is
necessary to compare the test glycosylated leptin
protein to known proteins used to calibrate the gel
filtration column. The methods as in the Gel
Filtration Calibration Kit Instruction Manual,

25   (Pharmacia Biotech paper 11-B-033-07, Rev.2), are
herein incorporated by reference. Generally, selected
proteins of known Stokes' radius are filtered through
the column, and the fraction where each eluted is
noted. The fraction containing the subject

30   glycosylated leptin protein is compared to the fraction
of the calibrated proteins.

          Thus, the glycosylated leptin proteins are
those having a Stokes' radius (of the glycosylated

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leptin protein moiety alone, not including any chemical
derivatization which may be further performed, as
indicated below) of greater than or equal to 30 Å as
determined by gel filtration. The gel filtration may
5   be accomplished using dextran-coated agarose gel
filtration substances, such as SuperDex™ or
Sepharcryl®, as described above. The buffer may be
phosphate buffered saline.

Leptin Amino Acid Sequences
10

            1. Glycosylation sites. Generally, to
prepare the present glycosylated leptin composition,
one will begin with a selected amino acid sequence, and
modify that sequence to include the addition of sites
15 for N-linked or O-linked glycosylation. The following
formula is preferred for adding sites for N-linked
glycosylation (see generally, Creighton, Proteins, W.H.
Freeman and Company, N.Y., (1984) p. 498, plus index at
pages 76-78, incorporated herein by reference):
20   N - X - T/S
wherein "N" is Asparagine "X" is any amino acid except
proline and "T/S" is Threonine or Serine. Preferred is
the formula "N - X - T"; whereby the alteration with
respect to a starting leptin amino acid sequence is
25 that "X" remains the same as that for starting leptin
sequence (preferably SEQ. ID. NOS. 1 or 2, infra), and
the amino acid immediately downstream (toward the C-
terminus) is threonine. N-linked sites at the outer
surface of the protein are preferred. Surface residues
30 suitable for glycosylation can be identified by
examination of a three dimensional structure or model,
or by nuclear magnetic resonance or crystal structure
(as discussed below). Also, it has been determined
that a proline at position -1 with respect to the

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asparagine residue (i.e., toward the N-terminus) in
some glycosylation sites may be detrimental, and one
may seek to avoid a proline residue at such a site.
Working Examples 5 and 6 demonstrate the effect of

5   glycosylation site occupancy of N - X - S versus N - X
- T and adjacent amino acids.

O-linked glycosylation sites are found on the
outer surface of proteins generally near or adjacent to
proline residues. O-linked sites can be found or

10   introduced by including serine or threonine residues
near to or adjacent to proline residues. Generally,
threonine residues are preferred. For example, SEQ. ID
NO: 1 (below) has a proline at position 99, and a
threonine at position 100 was introduced. This leptin

15   was expressed in CHO cells and COS cells, and was O-
linked glycosylated.

In addition, one may select to combine N-
linked and O-glycosylation sites in the present
glycosylated leptin proteins. As described above, one

20   may add one or more O-linked glycosylation site, and,
in addition, add one or more N-linked glycosylation
sites.

2. Sites for Glycosylation. Generally, one
will modify the protein backbone using the above

25   formulas to add an N-linked or O-linked glycosylation
site.

In order to select a site along the protein
backbone for N-glycosylation, the general rule is that
the asparagine residue must be located on an external

30   surface of the protein to be available for adding the
carbohydrate moiety. For example, with respect to the
three dimensional structure of leptin, the asparagine
residue should be on a loop, β-turn, or on an outer

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surface of an alpha helix. This analysis is based on
the current structure of leptin and the structure
functional relationship of several cytokines.

When selecting the site for glycosylation,
5 one may consider the leptin's three dimensional
conformation. The first several amino acids of leptin
are disordered, which indicates a certain amount of
flexibility. Topologically, the leptin structure
(see, Zhang et al., Nature 387: 206-209 (1997)
10 (reporting the crystal structure of obese protein
leptin E-100, herein incorporated by reference)) is
similar to the structure of the cytokine, granulocyte
colony stimulating factor ("G-CSF")(see, e.g., U.S.
Patent No. 5,581,476, Osslund, disclosing the 3-D
15 structure of crystalline rmetHuG-CSF).

Given the apparent flexibility and apparent
lack of biological significance of helix A, one may
choose to modify SEQ. ID NO: 1 to include glycosylation
sites at residues Val1 or Pro2.
20 Asp23 (of SEQ. ID NO: 1)is on the last turn
of helix A and is considered a good choice as the side
chain is at least partially on the outer surface of the
protein.

The proline residue at position 47 and the
25 isoleucine residue at position 48 (of SEQ. ID NO: 1)
are at the end of the AB loop, only a couple of
residues from the beginning of Helix B. They are on the
surface of the protein, and may be suitable for
glycosylation site insertion.
30 The proline residue at position 69 is on the
surface of the protein, which is a good position for
glycosylation.

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The phenylalanine residue at position 92 is at the end of the C helix and its side chain is facing opposite to which may be the receptor binding face. This is likely to yield the best result in that there
5   is the least interference from any glycosylation moiety with receptor binding.

Serine at position 102 is at the protein surface in the middle of the CD loop and should be in a relatively flexible portion of the structure, along
10   with positions 101(alanine) and 103(glycine).

Thus, the present invention relates to a glycosylated leptin protein comprising SEQ. ID NO: 1 (rHu-Leptin 1-146, below) or SEQ. ID NO: 2 (rHu-Leptin 1-145, below) having one or more sequence alterations
15   as a site of glycosylation. Said sequence alterations may be selected from among:

01V->N 02P->A 03I->T or S (i.e., altering the first amino acid in SEQ. ID NO: 1, below, which is a valine, to asparagine, altering the second amino acid
20   from proline to any of the other 19 amino acids (such as alanine), and altering the third amino acid from isoleucine to threonine or serine);

02P->N 03I 04Q->T or S (i.e., altering the second amino acid in SEQ. ID NO: 1, below, which is a
25   proline, to asparagine, maintaining the third amino acid as isoleucine, and altering the fourth amino acid from glutamine to threonine or serine);

23D->N 24I 25S->T or maintain as S (i.e., alter the 23rd amino acid in SEQ. ID NO: 1, below,
30   which is an aspartic acid to asparagine, maintaining the 24th amino acid as isoleucine, and for the 25th amino acid, either maintaining as serine or changing to threonine);

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47P->N 48I 49L->T or S (<u>i.e.</u>, altering the
47th amino acid from proline to asparagine, maintaining
the 48th amino acid as isoleucine, and altering the 49th
amino acid from leucine to threonine or serine);

5   48I->N 49L 50T or T->S (<u>i.e.</u>, altering the
48th amino acid from isoleucine to asparagine,
maintaining the 49th amino acid as leucine, and
maintaining the 50th amino acid as threonine, or
altering to serine);

10   69P->N 70S 71R->T or S (<u>i.e.</u>, altering the
69th amino acid in SEQ. ID NO: 1, below, from proline
to asparagine, maintaining the 70th amino acid as
serine, and altering the 71st amino acid from arginine
to threonine);

15   92F->N 93S 94K->T or S (<u>i.e.</u>, altering the
92nd amino acid of SEQ. ID NO: 1, below, from
phenylalanine to asparagine, maintaining the 93rd amino
acid as a serine, and altering the 94th amino acid from
lysine to threonine or serine);

20   101A->N 102S 103G->T or S (<u>i.e.</u>, altering the
101st amino acid in SEQ. ID NO: 1, below, from alanine
to asparagine, maintaining the 102nd amino acid as
serine, and altering the 103rd amino acid from glycine
to threonine or serine).

25   102S->N 103G 104L->T or S (<u>i.e.</u>, altering the
102nd amino acid in SEQ. ID NO: 1, below, from
tryptophan to asparagine, maintaining the 103rd amino
acid as glycine, and altering the 104th amino acid from
leucine to threonine or serine).

30   103G->N 104 L 105E->T or S (<u>i.e.</u>, altering
the 103rd amino acid in SEQ. ID NO: 1, below, from
glycine to asparagine, maintaining the 104th amino acid

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as leucine, and altering the 105th amino acid from
glutamic acid to threonine or serine).

Thus, the above shorthand notations indicate
the amino acid location with respect to SEQ. ID NO: 1,
and the change from one amino acid ---> to another
amino acid. As indicated below, the change in the
third amino acid (the amino acid toward the C-terminus
of the protein) to threonine is preferred for ease in
commercial manufacture, particularly in glycosylation
efficiency, although, as indicated above, a serine may
also be used at this location. Conventional single
letter amino acid abbreviations are used, as in Stryer,
Biochemistry, Third Edition (1988), W.H. Freeman and
Company, New York, inside back cover, herein
incorporated by reference.

In view of above, the present invention also
relates to a glycosylated leptin protein comprising
SEQ. ID NO: 1 (rHu-Leptin 1-146, below) having one or
more sequence alterations as a site of glycosylation
selected from among (where "T/S" denotes threonine or
serine):

    (a) 01V->N 02P->X (where X is any amino acid
    except proline) 03I->T/S
        (b)   02P->N 03I 04Q->T/S
        (c)   23D->N 24I 25S->T/S
        (d)   47P->N 48I 49L->T/S
        (e)   48I->N 49L 50T/S
        (f)   69P->N 70S 71R->T/S
        (g)   92F->N 93S 94K->T/S
        (h) 101A->N 102S 103G->T/S
        (i) 102S->N 103G 104L->T/S
        (j) 103G->N 104L 105E->T/S

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The working examples below demonstrate
biological activity which at least approximates non-
glycosylated rmetHu-Leptin 1-146 (SEQ. ID NO: 1) for
single- and double-glycosylation site leptin proteins.

5   Moreover, particular three, four and five-glycosylation
site leptin proteins have demonstrated increased
biological activity. Thus, the present invention also
includes particular glycosylated leptin proteins as set
forth in the working examples:

10   --a glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having a
glycosylation site located at a position selected from
among (with respect to the numbering of SEQ. ID NO: 1:
1, 2, 4, 8, 23, 44, 47, 48, 69, 70, 93, 97, 100, 101,

15   102, 103, 118 and 141.

             --a glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having two
glycosylation sites, said two sites selected from among
(with respect to the numbering of SEQ. ID NO: 1):

20   47 + 69;

     48 + 69;

     69 + 101;

     69 + 102;

     69 + 103;

25   69 + 118; and,

     100 + 102.

             --a glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having three
glycosylation sites, said three sites selected from

30   among (with respect to the numbering of SEQ. ID NO: 1):

     2 + 47 + 69

     23 + 47 + 69;

     47 + 69 + 100;

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```
47 + 69 + 102;
48 + 69 + 118;
69 + 102 + 118;  and,
69 + 103 + 118.
```

--a glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having four
glycosylation sites, said four sites selected from
among (with respect to the numbering of SEQ. ID NO: 1):

```
2 + 47 + 69 + 92;
2 + 47 + 69 + 102;
23 + 47 + 69 + 92;
23 + 47 + 69 + 102; and,
47 + 69 + 100 + 102.
```

--a glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having five
glycosylation sites, said five sites selected from
among (with respect to the numbering of SEQ. ID NO: 1):

```
2 + 23 + 47 + 69 + 92
2 + 47 + 69 + 92 + 102
23 + 47 + 69 + 92 + 102.
```

More particularly, the present invention
includes the following glycosylated leptin protein
amino acid sequences, DNAs encoding such sequences, and
specific DNAs as set forth below:

Glycosylated leptin 2,47,69 (SEQ. ID NO: 25, DNA):

```
  1 GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51 TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101 AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151 TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201 TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251 GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG
301 GCCAGTGGCC TGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351 AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
```

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```
      401  AGGACATGCT GTGGCAGCTG GACCTAAGCC CTGGGTGC
```

Glycosylated leptin 2,47,69 (SEQ. ID NO: 26, protein):

```
        1  VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
       51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPW
      101  ASGLETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

Glycosylated leptin 2,47,69,92 (SEQ. ID NO: 27, DNA):

```
        1  GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
       51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
      101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GCTCCACAA CATCACGACC
      151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
      201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
      251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG
      301  GCCAGTGGCC TGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
      351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
      401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

Glycosylated leptin 2,47,69,92 (SEQ. ID NO: 28, protein)

```
        1  VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
       51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPW
      101  ASGLETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

Glycosylated leptin 2,47,69,102 (SEQ. ID NO: 29, DNA):

```
        1  GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
       51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
      101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GCTCCACAA CATCACGACC
      151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
      201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
      251  GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG
      301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
      351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
      401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

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Glycosylated leptin 2,47,69,102 (SEQ. ID NO: 30 protein):

```
  1  VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPW
101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

Glycosylated leptin 47,69,102 (SEQ. ID NO: 31, DNA):

```
  1  GTGCCCATCC AAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG
301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

Glycosylated leptin 47,69,102 (SEQ. ID NO: 32, protein)

```
  1  VPIQKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPW
101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

Glycosylated leptin 2,47,69,92,102 (SEQ. ID NO: 33, DNA):

```
  1  GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG
301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

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Glycosylated leptin 2,47,69,92,102 (SEQ. ID NO: 34, protein):

```
  1  VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT

 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPW

101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

Glycosylated leptin 47,69,92,102 (SEQ. ID NO: 35, DNA):

```
  1  GTGCCCATCC AAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT

 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC

101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC

151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG

201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC

251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG

301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC

351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC

401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

Glycosylated leptin 47,69,92,102 (SEQ. ID NO: 36, protein):

```
  1  VPIQKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT

 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPW

101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

These were the specific amino acid sequences and corresponding DNAs used in the working examples below.

Characterization by Sialic Acid Moieties

Furthermore, the present glycosylated proteins may be characterized by their number of sialic acid moieties. Generally, there may be zero to four sialic acid moieties at an N-linked glycosylation site, and zero to two sialic acid moieties at an O-linked glycosylation site. A typical glycosylated protein preparation will contain a mixture of fully (i.e.,

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having a sialic acid moiety occupying all available
sites) and partially (_i.e._, having a sialic acid moiety
occupying less than all available sites) sialated
glycosylated proteins molecules.

5   The number of sialic acid moieties may be
determined by methods available to those skilled in the
art. For example, one may measure the molecular weight
of the protein or preparation thereof before and after
treatment with enzymes which remove sialic acid, and

10   calculate the molecular weight of the constituents.
Alternatively, one may use isoelectric focusing or
other methods to determine the sialic acid content.
          For example, human leptin 1-145 (SEQ. ID NO:
2, below) contains two O-linked glycosylation sites,

15   and thus, when fully sialated, four sialic acid
moieties. The present working examples with a single N-
linked glycosylation site contain four sialic acid
moieties, when fully sialated. The two-site
glycosylated leptin proteins below, when fully

20   glycosylated, contain 8 sialic acid moieties, the three
site, 12 sialic acid moieties, the four, 16 sialic acid
moieties, and the five site glycosylated leptins, 20
sialic acid moieties. The present invention thus
encompasses glycosylated leptin protein preparation

25   wherein each glycosylated leptin protein molecule in
said preparation has five or more sialic acid moieties.
More preferably, for purposes of enhancing a sustained
release effect of a therapeutic protein, the present
invention also encompasses a glycosylated leptin

30   protein preparation wherein each glycosylated leptin
protein molecule in said preparation has 8 to 20 sialic
acid residues. One may also choose to add additional

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glycosylation sites, and increase the sialic acid
content above 20 accordingly.

   3.   Leptin Protein Backbone. The type of
leptin used for the glycosylated leptin pharmaceutical
5 compositions may be selected from those described in
PCT International Publication Number WO96/05309, as
cited above and herein incorporated by reference in its
entirety. Figure 3 of that publication (as cited
therein SEQ. ID NO: 4) depicts the full deduced amino
10 acid sequence derived for human leptin (also referred
to as the human "OB" protein). The amino acids are
numbered from 1 to 167. A signal sequence cleavage
site is located after amino acid 21 (Ala) so that the
mature protein extends from amino acid 22 (Val) to
15 amino acid 167 (Cys). For the present disclosure, a
different numbering is used herein, where the amino
acid position 1 is the valine residue which is at the
beginning of the mature protein.

   The amino acid sequence for mature,
20 recombinant human leptin is presented herein as SEQ. ID
NO: 1, where the first amino acid of the mature protein
is valine (at position 1) (herein called rHu-Leptin 1-
146, SEQ. ID NO: 1):

			V	P	I	Q	K	V	Q	D	D	T	K	T	L	I	K	T	I	V
25	T	R	I	N	D	I	S	H	T	Q	S	V	S	S	K	Q	K	V	T	G
	L	D	F	I	P	G	L	H	P	I	L	T	L	S	K	M	D	Q	T	L
	A	V	Y	Q	Q	I	L	T	S	M	P	S	R	N	V	I	Q	I	S	N
	D	L	E	N	L	R	D	L	L	H	V	L	A	F	S	K	S	C	H	L
	P	W	A	S	G	L	E	T	L	D	S	L	G	G	V	L	E	A	S	G
30	Y	S	T	E	V	V	A	L	S	R	L	Q	G	S	L	Q	D	M	L	W
	Q	L	D	L	S	P	G	C												

   Alternatively, one may use a natural variant of
human leptin, which has 145 amino acids, and, as compared
to rHu-Leptin 1-146, has a glutamine absent at position

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28, presented below (herein called rHu-Leptin 1-145, SEQ.
ID NO: 2, wherein the blank ("_") indicates no amino
acid):

```
        V  P  I  Q  K  V  Q  D  D  T  K  T  L  I  K  T  I  V
 5   T  R  I  N  D  I  S  H  T  _  S  V  S  S  K  Q  K  V  T  G
     L  D  F  I  P  G  L  H  P  I  L  T  L  S  K  M  D  Q  T  L
     A  V  Y  Q  Q  I  L  T  S  M  P  S  R  N  V  I  Q  I  S  N
     D  L  E  N  L  R  D  L  L  H  V  L  A  F  S  K  S  C  H  L
     P  W  A  S  G  L  E  T  L  D  S  L  G  G  V  L  E  A  S  G
10   Y  S  T  E  V  V  A  L  S  R  L  Q  G  S  L  Q  D  M  L  W
     Q  L  D  L  S  P  G  C
```

For example, for the specific glycosylated
leptin proteins recited herein, one may choose to use
15 the "Q-" version of human leptin (1-145, SEQ. ID NO: 2)
and modify the corresponding sites enumerated for the
1-146 amino acid human leptin to include glycosylation
sites.

Generally, the leptin protein for use herein
20 will be capable of therapeutic use in humans (see also,
animal leptins, below). Thus, one may empirically test
activity to determine which leptin protein forms may be
used. As set forth in WO96/05309, leptin protein in
its native form, or fragments (such as enzyme cleavage
25 products) or other truncated forms and analogs may all
retain biological activity. Any of such forms may be
used to prepare the present glycosylated leptin
compositions, although such altered forms should be
tested to determine desired characteristics. See also,
30 PCT International Publication Numbers WO96/40912,
WO97/06816, WO97/18833, WO97/38014 and WO98/08512, all
here incorporated by reference.

One may prepare an analog of recombinant
human leptin by altering amino acid residues in the
35 recombinant human sequence, such as substituting the

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amino acids which diverge from the murine sequence.
Murine leptin is substantially homologous to human
leptin, particularly as a mature protein and, further,
particularly at the N-terminus. Because the
5   recombinant human protein has biological activity in
mice, such an analog would likely be active in humans.
For example, in the amino acid sequence of native human
leptin as presented in SEQ. ID NO: 1, one may
substitute with another amino acid one or more of the
10   amino acids at positions 32, 35, 50, 64, 68, 71, 74,
77, 89, 97, 100, 105, 106, 107, 108, 111, 118, 136,
138, 142, and 145. One may select the amino acid at
the corresponding position of the murine protein (SEQ.
ID NO: 3) or another amino acid.

15   One may further prepare synthetic molecules
based on the rat leptin, called, OB protein, sequence.
Murakami et al., _Biochem. Biophys. Res. Comm._ 209:
944-52 (1995) herein incorporated by reference. Rat OB
protein differs from human OB protein at the following
20   positions (using the numbering of SEQ. ID NO:: 1):   4,
_32_, 33, _35_, _50_, 68, _71_, _74_, _77_, 78, _89_, _97_, _100_, 101,
102, _105_, _106_, _107_, _108_, _111_, _118_, _136_, _138_ and _145_.
One may substitute with another amino acid one or more
of the amino acids at these divergent positions. The
25   positions in bold print are those in which the murine
OB protein as well as the rat OB protein are divergent
from the human OB protein and, thus, are particularly
suitable for alteration. At one or more of the
positions, one may substitute an amino acid from the
30   corresponding rat OB protein, or another amino acid.
          The positions from both rat and murine OB
protein which diverge from the mature human OB protein
are: 4, 32, 33, 35, 50, 64, 68, 71, 74, 77, 78, 89,
97, 100, 102, 105, 106, 107, 108, 111, 118, 136, 138,
35   142, and 145. An OB protein according to SEQ. ID NO::

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1 having one or more of the above amino acids replaced
with another amino acid, such as the amino acid found
in the corresponding rat or murine sequence, may also
be effective.

5   In addition, the amino acids found in rhesus
monkey OB protein which diverge from the mature human
OB protein are (with identities noted in parentheses in
one letter amino acid abbreviation): 8 (S), 35 (R), 48
(V), 53 (Q), 60 (I), 66 (I), 67 (N), 68 (L), 89 (L),

10   100 (L), 108 (E), 112 (D) and 118 (L). Since the
recombinant human OB protein is active in cynomolgus
monkeys, a human OB protein according to SEQ. ID NO: 1
having one or more of the rhesus monkey divergent amino
acids replaced with another amino acid, such as the

15   amino acids in parentheses, may be effective. It
should be noted that certain rhesus divergent amino
acids are also those found in the above murine species
(positions 35, 68, 89, 100 and 112). Thus, one may
prepare a murine/rhesus/human consensus molecule (using

20   the numbering of SEQ. ID NO: 1) having one or more of
the amino acids at positions replaced by another amino
acid: 4, 8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68,
71, 74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108,
111, 112, 118, 136, 138, 142 and 145.

25   Other analogs may be prepared by deleting a
part of the protein amino acid sequence. For example,
the mature protein lacks a signal sequence (-22 to -1).
One may delete a portion of the mature protein, and
this deletion may be incident to manufacture, e.g.,

30   cleavage of signal peptide or other presequences beyond
the first N-terminal amino acid of the mature protein.
Also, the N-terminus may contain one or more additional
amino acids, which may be incident to use of such
presequences, such as, for example, cleavage in the

35   middle of a signal peptide cleavage site, so that a

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portion of the amino acids of the cleavage site is attached.

One may prepare the following truncated forms of human leptin protein molecules (using the numbering
5 of SEQ. ID NO: 1):

(a) amino acids 98-146;

(b) amino acids 1-99 and (connected to) 112-146;

(c) amino acids 1-99 and (connected to)
10 112-146 having one or more of amino acids 100-111 sequentially placed between amino acids 99 and 112.

In addition, the truncated forms may also have altered one or more of the amino acids which are divergent (in the murine, rat or rhesus OB protein)
15 from human OB protein. Furthermore, any alterations may be in the form of altered amino acids, such as peptidomimetics or D-amino acids.

Included are those proteins as set forth above with amino acid substitutions which are
20 "conservative" according to acidity, charge, hydrophobicity, polarity, size or any other characteristic known to those skilled in the art. These are set forth in Table 1, below. See generally, Creighton, Proteins, W.H. Freeman and Company, N.Y.,
25 (1984) p. 498, plus index, passim. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is herein incorporated by reference.

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Table 1
Conservative Amino Acid Substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:-	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

5   Therefore, the present glycosylated human
leptin proteins may be prepared by first starting with
a sequence selected from among (according to the amino
acid sequence as presented in SEQ. ID NO:: 1 herein):
                (a) the amino acid sequence of SEQ. ID
10 NO:: 1, optionally lacking a glutaminyl residue at
position 28;
                (b) an amino acid sequence of subpart
(a) having a different amino acid substituted in one or
more of the following positions: 4, 8, 32, 33, 35, 48,
15 50, 53, 60, 64, 66, 67, 68, 71, 74, 77, 78, 89, 97,
100, 102, 105, 106, 107, 108, 111, 112, 118, 136, 138,
142 and 145;

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(c) a truncated leptin protein analog
selected from among: (using the numbering of subpart
(a) above):

          (i)   amino acids 98-146

5   (ii)   amino acids 1-99 and 112-146

          (iii) amino acids 1-99 and 112-146
having one or more of amino acids 100-111
sequentially placed between amino acids 99 and
112; and,

10   (iv)   the truncated leptin analog of
subpart (i) having one or more of amino acids 100,
102, 105, 106, 107, 108, 111, 112, 118, 136, 138,
142 and 145 substituted with another amino acid;

                (v)   the truncated leptin analog of

15   subpart (ii) having one or more of amino acids 4,
8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71,
74, 77, 78, 89, 97, 112, 118, 136, 138, 142 and
145 replaced with another amino acid;

                (vi)   the truncated leptin analog of

20   subpart (iii) having one or more of amino acids 4,
8, 32, 33, 35, 48, 50, 53, 60, 64, 66, 67, 68, 71,
74, 77, 78, 89, 97, 100, 102, 105, 106, 107, 108,
111, 112, 118, 136, 138, 142 and 145 replaced with
another amino acid; and

25   (d)   a leptin protein of any of subparts (a)-
(c) having one or more conserved amino acids
substitutions, and then selecting a site, preferably on
the external surface of an alpha helix, to insert, by
addition or substitution, a glycosylation site.
30   Particular glycosylation sites are recited _supra_.

     Leptin proteins, analogs and related
molecules are also reported in the following
publications; however, no representation is made with
35   regard to the activity of any composition reported:

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U.S. Patent Nos. 5,521,283; 5,525,705;
5,532,336; 5,552,522; 5,552,523; 5,552,524;
5,554,727; 5,559,208; 5,563,243; 5,563,244;
5,563,245; 5,567,678; 5,567,803; 5,569,743;
5,569,744; 5,574,133; 5,580,954; 5,594,101;
5,594,104; 5,605,886; 5,614,379; 5,691,309;
5,719,266 (all assigned to Eli Lilly and
Company);
PCT WO96/23513; WO96/23514; WO96/23515;
WO96/23516; WO96/23517; WO96/23518;
WO96/23519; WO96/23520; WO96/23815;
WO96/24670; WO96/27385; EP 725078; EP 725079
(all assigned to Eli Lilly and Company);
PCT WO96/22308 (assigned to Zymogenetics);
PCT WO96/29405 (assigned to Ligand
        Pharmaceuticals, Inc.);
PCT WO96/31526 (assigned to Amylin
        Pharmaceuticals, Inc.);
PCT WO96/34885 (assigned to Smithkline
Beecham   PLC);
PCT WO96/35787 (assigned to Chiron);
EP 736599 (assigned to Takeda);
EP 741187 (assigned to F. Hoffman LaRoche).

    To the extent these references provide for
useful leptin proteins or analogs, or associated
compositions or methods, such compositions and/or
methods may be used in conjunction with the present
glycosylated leptin pharmaceutical compositions, such
as for co-administration (together or separately, in a
selected dosage schedule). With the above provisos,
these publications are herein incorporated by
reference.

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Nucleic Acids, Vectors, Host Cells and other Expression Systems

Also comprehended by the present invention are nucleic acids encoding the present glycosylated
5 leptin proteins. Such nucleic acids may be prepared by site directed mutagenesis of an existing nucleic acid sequence or by synthetic means, or by other means as are available to those skilled in the art. Methods as disclosed in the Reference Examples below are
10 illustrative.

Vectors include plasmidic as well as viral vectors as are available to those skilled in the art. Vectors may be for cloning or expression, and include plasmids, cosmids, and prokaryotic or eukaryotic-cell
15 infecting viruses. For expression of glycosylated protein, vectors will be useful for expression in a eukaryotic cell. The expression system may be constitutive or inducible, such as systems including an inducible mouse mammary tumor virus LTR promoter.
20 Enhancers, transcription terminators, splice donor and acceptor sites, and other elements may be included in the overall system as is known to those skilled in the art.

Vectors disclosed in the Reference Examples
25 below are illustrative. The present working examples used a modified form of pDSRα2, to express glycosylated leptin proteins.

The host cells may be prokaryotic, such as bacteria used for cloning of the present nucleic acids,
30 for example. Other host cells may be eukaryotic. Eukaryotic host cells may be selected from the phylum Chordata, such as those in the class Mammalia. Primate cells, including human cells (such as Namalwa, HeLa, human hepatocellular carcinoma, such as Hep G2 cells,
35 human embryonic kidney cells, human liver cells, human

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lung cells or cells cultured from human sources) and
COS cells, or other mammalian cells, such as baby
hamster kidney cells ("BHK" cells), Chinese hamster
ovary cells ("CHO") cells, mouse sertoli cells, canine
5   kidney cells, buffalo rat liver cells, mouse mammary
tumor cells may be used. Insect cells may also be
used. Lesser host cell organisms, such as yeasts, and
fungi, are also included. See generally, Margulis,
Five Kingdoms, 2d Edition (1988) W.H. Freeman & Co.,
10   New York, for classifications of organisms.

          One may seek to co-express more than one
desired protein. For example, the present glycosylated
leptin protein may be expressed in a eukaryotic host
cell along with one or more other desired proteins. The
15   proteins may be separated using a number of available
separation techniques, depending on the characteristics
of the protein. For example, one may, in a single host
cell, such as a CHO cell, express a glycosylated leptin
protein, as well as a different protein, such as a
20   different glycosylated protein desired for therapeutic
use. One may use, for example, molecular weight to
separate the proteins for purification. In this way,
economies of manufacture may be achieved, by producing
two different proteins from a single cell culture.

25   One may also use transgenic animals to
express the present glycosylated leptin protein. For
example, one may use a transgenic milk-producing animal
(a cow or goat, for example) and obtain the present
glycosylated leptin protein in the milk produced. One
30   may use plants to produce the present glycosylated
proteins, however, in general, the glycosylation
occurring in plants is different from that produced in
mammalian cells, and may result in a glycosylated

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product which is not suitable for human therapeutic
use.

<u>Gene Therapy</u>

The DNA provided herein (or
5   corresponding RNAs) may also be used for gene therapy.
A review article on gene therapy is Verma, Scientific
American, November 1990, pages 68-84 which is herein
incorporated by reference.

Thus, the present invention provides for a
10 population of cells expressing the present glycosylated
leptin protein. Such cells are suitable for
transplantation or implantation into an individual for
therapeutic purposes. One may then implant such cells
into an individual. Such cells may, for example, be
15 liver cells, bone marrow cells, or cells derived from
umbilical cord. Alternatively, one may wish to use
circulating cells such as blood progenitor cells, T
cells or other blood cells. For humans, human cells may
be used. Cells may be in the form of tissue. Such
20 cells may be cultured prior to transplantation or
implantation.

The cells to be transferred to the recipient
may be cultured using one or more factors affecting the
growth or proliferation of such cells if appropriate.
25 Hematopoietic factors may be used in culturing
hematopoietic cells. Such factors include G-CSF, EPO,
MGDF, SCF, Flt-3 ligand, interleukins (e.g., IL1-IL13),
GM-CSF, LIF, and analogs and derivatives thereof as
available to one skilled in the art.
30   Nerve cells, such as neurons or glia, may
also be used, and these may be cultured with
neurotrophic factors such as BDNF, CNTF, GDNF, NT3, or
others.

Techniques for the encapsulation of living
35 cells are familiar to those of ordinary skill in the

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art, and the preparation of the encapsulated cells and
their implantation in patients may be accomplished
without undue experimentation. For example, Baetge et
al. (International Publication No. WO 95/05452;
5 International Application No. PCT/US94/09299 the
disclosure of which is hereby incorporated by
reference) describe membrane capsules containing
genetically engineered cells for the effective delivery
of biologically active molecules. The capsules are
10 biocompatible and are easily retrievable. The capsules
encapsulate cells transfected with recombinant DNA
molecules comprising DNA sequences coding for
biologically active molecules operatively linked to
promoters that are not subject to down regulation in
15 vivo upon implantation into a mammalian host. The
devices provide for the delivery of the molecules from
living cells to specific sites within a recipient. In
addition, see U.S. Patent Numbers 4,892,538, 5,011,472,
and 5,106,627, each of which is specifically
20 incorporated herein by reference. A system for
encapsulating living cells is described in PCT
Application WO 91/10425 of Aebischer et al.,
specifically incorporated herein by reference. See
also, PCT Application WO 91/10470 of Aebischer et al.,
25 Winn et al., Exper. Neurol. 113:322-329(1991),
Aebischer et al., Exper. Neurol. 111:269-275, (1991);
Tresco et al., ASAIO 38:17-23(1992), each of which is
specifically incorporated herein by reference.

        In vivo and in vitro gene therapy delivery of
30 the present glycosylated leptin protein is also
envisioned. In vivo gene therapy may be accomplished
by introducing the nucleic acid encoding a present
glycosylated leptin protein into cells via local
injection of a polynucleotide molecule or other
35 appropriate delivery vectors. (Hefti, J.
Neurobiology,. 25:1418-1435, 1994). For example, a

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polynucleotide molecule encoding a glycosylated leptin
protein may be contained in an adeno-associated virus
vector for delivery into the targeted cells (e.g.,
Johnson, International Publication No. WO 95/34670;
5   International Application No. PCT/US95/07178 the
disclosure of which is hereby incorporated by
reference). The recombinant adeno-associated virus
(AAV) genome contains AAV inverted terminal repeats
flanking a DNA sequence encoding the neurotrophic
10   factor operably linked to functional promoter and
polyadenylation sequences.
          Alternative viral vectors include, but are
not limited to, retrovirus, adenovirus, herpes simplex
virus and papilloma virus vectors. U.S. 5,672,344
15   (issued September 30, 1997, Kelley et al., University
of Michigan), the disclosure of which is hereby
incorporated by reference, describes an in vivo viral-
mediated gene transfer system involving a recombinant
neurotropic HSV-1 vector. U.S. 5,399,346 (issued March
20   21, 1995, Anderson et al., Department of Health and
human Services), the disclosure of which is
incorporated by reference herein, provides examples of
a process for providing a patient with a therapeutic
protein by the delivery of human cells which have been
25   treated in vitro to insert a DNA segment encoding a
therapeutic protein. Additional methods and materials
for the practice of gene therapy techniques, the
disclosures of which are incorporated by reference
herein, are described in U.S. 5,631,236 (issued May 20,
30   1997, Woo et al., Baylor College of Medicine) involving
adenoviral vectors; U.S. 5,672,510 (issued September
30, 1997, Eglitis et al., Genetic Therapy, Inc.)
involving retroviral vectors; and U.S. 5,635,399
(issued June 3, 1997, Kriegler et al., Chiron
35   Corporation) involving retroviral vectors expressing
cytokines.

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Nonviral delivery methods include liposome-
mediated transfer, naked DNA delivery (direct
injection), receptor-mediated transfer (ligand-DNA
complex), electroporation, calcium phosphate
5   precipitation and microparticle bombardment (e.g., gene
gun). Gene therapy materials and methods may also
include inducible promoters, tissue-specific enhancer-
promoters, DNA sequences designed for site-specific
integration, DNA sequences capable of providing a
10   selective advantage over the parent cell, labels to
identify transformed cells, negative selection systems
and expression control systems (safety measures), cell-
specific binding agents (for cell targeting), cell-
specific internalization factors, transcription factors
15   to enhance expression by a vector as well as methods of
vector manufacture. Such additional methods and
materials for the practice of gene therapy techniques,
the disclosures of which are incorporated by reference
herein, are described in U.S. 4,970,154 (issued
20   November 13, 1990, D.C. Chang, Baylor College of
Medicine) electroporation techniques; WO 9640958
(published 961219 , Smith et al., Baylor College of
Medicine) nuclear ligands; U.S. 5,679,559 (issued
October 21, 1997, Kim et al., University of Utah
25   Research Foundation) concerning a lipoprotein-
containing system for gene delivery; U.S. 5,676,954
(issued October 14, 1997, K.L. Brigham, Vanderbilt
University involving liposome carriers; U.S. 5,593,875
(issued January 14, 1997, Wurm et al., Genentech, Inc.)
30   concerning methods for calcium phosphate transfection;
and U.S. 4,945,050 (issued July 31, 1990, Sanford et
al., Cornell Research Foundation) wherein biologically
active particles are propelled at cells at a speed
whereby the particles penetrate the surface of the
35   cells and become incorporated into the interior of the
cells. Expression control techniques include chemical

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induced regulation (e.g., WO 9641865 and WO 9731899),
the use of a progesterone antagonist in a modified
steroid hormone receptor system (e.g., U.S. 5,364,791),
ecdysone control systems (e.g., WO 9637609), and
5   positive tetracycline-controllable transactivators
(e.g., U.S. 5,589,362; U.S. 5,650,298; and U.S.
5,654,168).

    It is also contemplated that the present gene
therapy or cell therapy can further include the
10 delivery of a second therapeutic composition. For
example, the host cell may be modified to express and
release both a glycosylated leptin protein and native
human leptin. Alternatively, they may be expressed in
and released from separate cells. Such cells may be
15 separately introduced into the patient or the cells may
be contained in a single implantable device, such as
the encapsulating membrane described above.

            Selective Binding Molecules

    The present invention also relates to
20 selective binding moieties of the present glycosylated
human leptin proteins. A "selective binding moiety"
denotes a substance which selectively binds to the
present glycosylated human leptin proteins, in
glycosylated or unglycosylated form. Selectivity is
25 determined by whether the binding moiety binds to the
subject leptin protein above background (nonselective)
levels. Particular examples of selective binding
moieties include antibodies, such as monoclonal,
polyclonal, monospecific polyclonal, produced by, for
30 example hybridoma technology or using recombinant
nucleic acid means. See, e.g., Huse et al., Science
246: 1275 (1989). Also comprehended herein are nucleic
acids, vectors, host cells and other materials and
methods used in the recombinant nucleic acid expression

of a selective binding moiety, such as a recombinant
antibody. One may attach detectable labels to such
selective binding moieties, such as chemiluminescent,
fluorescent, colorimetric, or radioactive, using

5   materials and methods available to those skilled in the
art. One may prepare assays, or kits, containing one
or more of these selective binding molecules, for
detection or measurement of the present leptin
proteins. Illustrative is a kit including monoclonal

10   antibodies selective for a particular glycosylated
leptin protein, and means to detect selective binding
of said monoclonal antibodies to said glycosylated
leptin protein. Other materials and methods for such
kits are available to those skilled in the art.

15   Formulations and Derivatives
            In yet another aspect of the present
invention, provided are methods of using pharmaceutical
compositions of the present glycosylated leptin
compositions, and derivatives (see below). Such

20   pharmaceutical compositions may be for administration
by injection, or for oral, intrathecal, pulmonary,
nasal, transdermal or other forms of administration.
In general, comprehended by the invention are
pharmaceutical compositions comprising effective

25   amounts of protein or derivative products of the
invention together with pharmaceutically acceptable
diluents, preservatives, solubilizers, emulsifiers,
adjuvants and/or carriers. Such compositions include
diluents of various buffer content (e.g., Tris-HCl,

30   acetate, phosphate), pH and ionic strength; additives
such as detergents and solubilizing agents (e.g., Tween
80, Polysorbate 80), anti-oxidants (e.g., ascorbic
acid, sodium metabisulfite), preservatives (e.g.,

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Thimersol, benzyl alcohol) and bulking substances
(e.g., lactose, mannitol); incorporation of the
material into particulate preparations of polymeric
compounds such as polylactic acid, polyglycolic acid,
5   etc. or into liposomes. See, e.g., PCT WO96/29989,
Collins et al., "Stable protein: phospholipid
compositions and methods," published October 3, 1996,
herein incorporated by reference. Hylauronic acid may
also be used, and this may have the effect of promoting
10   sustained duration in the circulation. Such
compositions may influence the physical state,
stability, rate of in vivo release, and rate of in vivo
clearance of the present proteins and derivatives.
See, e.g., Remington's Pharmaceutical Sciences, 18th
15   Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages
1435-1712 which are herein incorporated by reference.
The compositions may be prepared in liquid form, or may
be in dried powder, such as lyophilized form.
Implantable sustained release formulations are also
20   contemplated, as are transdermal formulations.
        Specifically contemplated are oral dosage
forms of the above derivatized proteins. Protein may
be chemically modified so that oral delivery of the
derivative is efficacious. Generally, the chemical
25   modification contemplated is the attachment of at least
one moiety to the protein (or peptide) molecule itself,
where said moiety permits (a) inhibition of
proteolysis; and (b) uptake into the blood stream from
the stomach or intestine. Also desired is the increase
30   in overall stability of the protein and increase in
circulation time in the body. See PCT WO95/21629,
Habberfield, "Oral Delivery of Chemically Modified
Proteins" (published August 17, 1995) herein

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incorporated by reference, and U.S. Patent No.
5,574,018, Habberfield et al., "Conjugates of Vitamin
B12 and Proteins," issued November 12, 1996, herein
incorporated by reference. The materials and methods
5 disclosed therein are applicable to the present
glycosylated leptin compositions and methods.

Also contemplated herein is pulmonary
delivery of the present protein, or derivative thereof.
The protein (derivative) is delivered to the lungs of a
10 mammal while inhaling and traverses across the lung
epithelial lining to the blood stream. See, PCT
WO94/20069, Niven et al., "Pulmonary administration of
granulocyte colony stimulating factor," published
September 15, 1994, herein incorporated by reference,
15 and PCT WO96/05309, previously incorporated by
reference at page 83 et seq., for example. The present
glycosylated leptin proteins may be spray-dried into
particles having an average size of less than 10
microns, or more preferably, 0.5 to 5 microns. Larger
20 sized particles may be used depending on the density of
each particle.

Nasal delivery of the protein (or analog or
derivative) is also contemplated. Nasal delivery
allows the passage of the protein to the blood stream
25 directly after administering the therapeutic product to
the nose, without the necessity for deposition of the
product in the lung. Formulations for nasal delivery
include those with absorption enhancing agents, such as
dextran or cyclodextran. Delivery via transport across
30 other mucous membranes is also contemplated.

The present glycosylated leptin proteins may
also be derivatized by the attachment of one or more
chemical moieties to the protein moiety. Chemical

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modification of biologically active proteins has been
found to provide additional advantages under certain
circumstances, such as increasing the stability and
circulation time of the therapeutic protein and
5   decreasing immunogenicity. See U.S. Patent
No. 4,179,337, Davis et al., issued December 18, 1979.
For a review, see Abuchowski et al., in Enzymes as
Drugs. (J.S. Holcerberg and J. Roberts, eds.
pp. 367-383 (1891)). A review article describing
10   protein modification and fusion proteins is Francis,
Focus on Growth Factors 3: 4-10 (May 1992) (published
by Mediscript, Mountview Court, Friern Barnet Lane,
London N20, OLD, UK). One may wish to further modify
the present glycosylated leptin compositions, such as
15   adding, by chemical modification, a water soluble
polymer. The addition of a chemical moiety will likely
require an additional manufacturing step, but may
result in further benefits in terms of improved product
characteristics (with the caveat that under some
20   conditions, chemical derivatization may make the
product less desirable, such as by inducing the
formation of kidney vacuoles, see supra). The chemical
moieties should be attached to the protein with
consideration of effects on functional or antigenic
25   domains of the protein. There are a number of
attachment methods available to those skilled in the
art. E.g., PCT WO96/11953, "N-Terminally Chemically
Modified Protein Compositions and Methods," published
April 25, 1996, herein incorporated by reference in its
30   entirety, and EP 0 401 384 herein incorporated by
reference (coupling PEG to G-CSF). The methods and
polymers disclosed therein in the above publications
are applicable to the present glycosylated leptin

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compositions, if derivatization is desired to further
improve characteristics of a therapeutic composition,
for example.

5   Fusion proteins may be prepared by attaching
polyaminoacids to glycosylated leptin protein moiety.
For example, the polyamino acid may be a carrier
protein which serves to further increase the
circulation half life of the protein. For the present
therapeutic or cosmetic purposes, such polyamino acid
10   should be those which do not create neutralizing
antigenic response, or other adverse response. Such
polyamino acid may be selected from the group
consisting of serum album (such as human serum
albumin), an antibody or portion thereof (such as an
15   antibody constant region, sometimes called "F_C") or
other polyamino acids. The location of attachment of
the polyamino acid may be at the N-terminus of the
glycosylated leptin protein moiety, or other place, and
also may be connected by a chemical "linker" moiety to
20   the protein. See, e.g., PCT WO 98/28427, published
July 2, 1998, entitled, "Ob Fusion Protein Compositions
and Methods", herein incorporated by reference in its
entirety. The polyamino acid may be used to aid in
detection or purification, such as using a "FLAG" tag,
25   "his" tag, "myc" tag or other polyamino acid tag known
to those skilled in the art.

        Relatedly, detectable labels may be attached
to the present glycosylated leptin proteins.
Radioisotopes, light-emitting (e.g., fluorescent or
30   chemiluminescent compounds), enzymatically cleavable
compounds, detectable antibody (or modification
thereof) or other substances may be used for such
labeling of the present proteins. Detecting protein via

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use of the labels may be useful for identifying the presence or amount of the present proteins, or a compound containing such proteins (such as an antibody/protein complex).

Dosages

One skilled in the art will be able to ascertain effective dosages by administration and observing the desired therapeutic effect. Presently, unmodified rmetHu-leptin 1-146 has been demonstrated to be effective at doses of 0.3 mg protein/kg body weight/day, and has been seen to be less effective at a dose of 0.1 mg protein/kg body weight/day. Greenberg et al., Preliminary safety and efficacy of recombinant methionyl human Leptin administered by SC injection in lean and obese subjects. Poster presented at: Annual Meeting of the American Diabetes Association; June 16, 1998, Chicago, IL. The desired dosage range, to have advantage over the existing rmetHu-leptin 1-146 is the same or lower than the above. Also, a desired dosage range may be one in which the same (or lower) protein load is administered less frequently. The effective dosages may be determined using diagnostic tools over time. For example, a diagnostic for measuring the amount of leptin in the blood (or plasma or serum) may first be used to determine endogenous levels of leptin. Such diagnostic tool may be in the form of an antibody assay, such as an antibody sandwich assay. The amount of endogenous leptin is quantified initially, and a baseline is determined. The therapeutic dosages are determined as the quantification of endogenous and exogenous leptin (that is, protein, analog or derivative found within the body, either self-produced

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or administered) is continued over the course of
therapy. The dosages may therefore vary over the
course of therapy, with a relatively high dosage being
used initially, until therapeutic or cosmetic benefit
5   is seen, and lower dosages used to maintain the
therapeutic or cosmetic benefits.

During an initial course of therapy of an
obese person, dosages may be administered whereby
weight loss and concomitant fat tissue decrease
10 increase is achieved. Once sufficient weight loss is
achieved, a dosage sufficient to prevent re-gaining
weight, yet sufficient to maintain desired weight or
fat mass may be administered. These dosages can be
determined empirically, as the effects of leptin are
15 reversible. E.g., Campfield et al., Science 269:
546-549 (1995) at 547. Thus, if a dosage resulting in
weight loss is observed when weight loss is not
desired, one would administer a lower dose, yet
maintain the desired weight.
20

Methods of Use
Therapeutic. Therapeutic uses include weight
modulation, the treatment or prevention of diabetes,
blood lipid reduction (and treatment of related
25 conditions), increasing lean body mass and increasing
insulin sensitivity. In addition, the present
compositions may be used for manufacture of one or more
medicaments for treatment or amelioration of the above
conditions.
30 Cosmetic. For those desiring solely
appearance enhancement, the present compositions may be
used for weight loss, or weight maintenance which has
no concomitant effect on an adverse medical condition.

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In addition, the present compositions may be used for manufacture of one or more preparations for cosmetic purposes.

Weight Modulation. The present compositions and methods may be used for weight reduction. Viewed another way, the present compositions may be used for maintenance of a desired weight or level of adiposity. As has been demonstrated in murine models (see supra), administration of the present glycosylated leptin proteins results in weight loss. The body mass lost is primarily of adipose tissue, or fat. Such weight loss, or maintenance of a particular weight, can be associated with the prevention or treatment of concomitant conditions, such as those below, and therefore constitute a therapeutic application.

Treatment of Diabetes. The present compositions and methods may be used in the prevention or treatment of Type I or Type II diabetes. As Type II diabetes can be correlated with obesity, use of the present invention to reduce weight (or maintain a desired weight, or reduce or maintain an adiposity level) can also alleviate or prevent the development of diabetes. Moreover, even in the absence of dosages sufficient to result in weight loss, the present compositions may be used to prevent or ameliorate diabetes.

Administration of the present compositions may result in an increased sensitivity to endogenous or exogenous insulin, and allow an individual to reduce or eliminate the amount administration of exogenous insulin required to treat type II diabetes. It is further contemplated that the present compositions may

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be used in the treatment, prevention or amelioration of
Type I diabetes.

Blood Lipid Modulation. The present
compositions and methods may be used in the modulation

5   of blood lipid levels. Ideally, in situations where
solely reduction in blood lipid levels is desired, or
where maintenance of blood lipid levels is desired, the
dosage will be insufficient to result in weight loss.
Thus, during an initial course of therapy of an obese

10 patient, dosages may be administered whereby weight
loss and concomitant blood lipid level lowering is
achieved. Once sufficient weight loss is achieved, a
dosage sufficient to prevent re-gaining weight, yet
sufficient to maintain desired blood lipid levels, or

15 other conditions as set forth herein, for example, may
be administered. Thus, if a dosage resulting in weight
loss is observed when weight loss is not desired, one
would administer a lower dose in order to achieve the
desired blood lipid levels, yet maintain the desired

20 weight. See, e.g., PCT Publication WO97/06816 herein
incorporated by reference.

Increasing Lean Mass or Insulin Sensitivity.
Ideally, in situations where solely an increase in lean
body mass is desired, the dosage will be insufficient

25 to result in weight loss. Thus, during an initial
course of therapy of an obese person, dosages may be
administered whereby weight loss and concomitant fat
tissue decrease/lean mass increase is achieved. Once
sufficient weight loss is achieved, a dosage sufficient

30 to prevent re-gaining weight, yet sufficient to
maintain desired lean mass increase (or prevention of
lean mass depletion) may be administered. For
increasing an individual's sensitivity to insulin,

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similar dosage considerations may be taken into account. Lean mass increase without weight loss may be achieved sufficient to decrease the amount of insulin (or, potentially, amylin, amylin antagonists or

5 agonists, or thiazolidinediones, or other potential diabetes treating drugs) an individual would be administered for the treatment of diabetes. For increasing overall strength, there may be similar dosage considerations. Lean mass increase with

10 concomitant increase in overall strength may be achieved with doses insufficient to result in weight loss. Other benefits, such as an increase in red blood cells (and oxygenation in the blood) and a decrease in bone resorption or osteoporosis may also be achieved in

15 the absence of weight loss. E.g., PCT WO97/18833, published May 29, 1997, herein incorporated by reference in its entirety.

Combination Therapies. The present compositions and methods may be used in conjunction

20 with other therapies, such as altered diet and exercise. Other medicaments, such as those useful for the treatment of diabetes (e.g., insulin and possibly amylin, antagonists or agonists thereof, thiazolidinediones, or other potential diabetes

25 treating drugs), cholesterol and blood pressure lowering medicaments (such as those which reduce blood lipid levels or other cardiovascular medicaments), activity increasing medicaments (e.g., amphetamines), diuretics (for liquid elimination), and appetite

30 suppressants (such as agents which act on neuropeptide γ receptors, serotonin reuptake inhibitors or gastric fat uptake inhibitors). Such administration may be simultaneous or may be in seriatim. In addition, the

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present methods may be used in conjunction with
surgical procedures, such as cosmetic surgeries
designed to alter the overall appearance of a body
(e.g., liposuction or laser surgeries designed to
5   reduce body mass, or implant surgeries designed to
increase the appearance of body mass). The health
benefits of cardiac surgeries, such as bypass surgeries
or other surgeries designed to relieve a deleterious
condition caused by blockage of blood vessels by fatty
10   deposits, such as arterial plaque, may be increased
with concomitant use of the present compositions and
methods. Methods to eliminate gall stones, such as
ultrasonic or laser methods, may also be used either
prior to, during or after a course of the present
15   therapeutic methods. Furthermore, the present methods
may be used as an adjunct to surgeries or therapies for
broken bones, damaged muscle, or other therapies which
would be improved by an increase in lean tissue mass.

            Methods of Manufacture

20   As indicated above, it has also been observed
that particular constructs of signal sequences and
mature protein sequences may improve glycosylation
efficiency. In this regard, the term "signal sequence"
(sometimes referred to in the art as "signal peptide")
25   is use to denote an peptide, found at or near the N-
terminus of the mature protein, usually about 15 to
about 30 amino acids long, rich in hydrophobic amino
acids, which facilitates secretion of the mature
protein into the endoplasmic reticulum. It is in the
30   endoplasmic reticulum, or cell membrane region, that
initial glycosylation of protein occurs. Signal
sequences are cleaved from the mature sequence prior to
the secretion of the mature protein. See Watson et

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al., Molecular Biology of the Gene, 4th Ed., 1987, at
page 731, (The Benjamin/Cummings Publishing Company,
Inc., Menlo Park, California) herein incorporated by
reference. In particular, various signal sequences
5   which are not-naturally found operably linked to a
naturally occurring leptin protein have been used, and
have been found to improve glycosylation efficiency of
multiply glycosylated leptin proteins.

For example, it has been found that, as
10 compared to the native human leptin signal sequence,
the signal sequence normally found connected to the
tissue plasminogen activator sequence, when used in
conjunction with the expression of various of the
multiply-glycosylated leptin proteins described herein,
15 results in higher levels of glycosylation (e.g.,
glycosylation moieties at all suitable sites in a
higher proportion of the expressed molecules of mature
protein).

Therefore, the present invention also relates
20 to a method of manufacturing a glycosylated leptin
protein comprising:

(a) culturing, under suitable conditions for
expression, a host cell containing a DNA sequence
encoding, in the 5' to 3' direction (i) a signal
25 sequence, and (ii) a DNA encoding a glycosylated leptin
protein; and

(b) obtaining said glycosylated leptin
protein.

Further, as discussed above, the present
30 invention relates to a method of manufacturing a
glycosylated leptin protein wherein said signal is
selected from among:

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a) (SEQ.ID NO. 3)(native human leptin signal peptide) MHWGTLCGFLWLWPYLFYVQA

(b) (SEQ.ID NO. 4)(modified human leptin signal peptide) MHWGTLCGFLWLWPYLFYVSPS

(c) (SEQ.ID NO. 5) (modified human leptin signal peptide) MHWGTLCGFLWLWPYLFYVSP

(d) (SEQ.ID NO. 6)(modified human leptin signal peptide) MHWGTLCGFLWLWPYLFYVSPA

(e) (SEQ.ID NO. 7)(modified human leptin signal peptide) MHWGTLCGFLWLWPYLFYVSNS

(f) (SEQ.ID NO. 8)(native human tPA signal peptide) MDAMKRGLCCVLLLCGAVFVSPS

(g) (SEQ.ID NO. 9)(native human tPA signal peptide) MDAMKRGLCCVLLLCGAVFVSP

(h) (SEQ.ID NO. 10)(modified tPA signal peptide) MDAMKRGLCCVLLLCGAVFVSNS

(i) (SEQ.ID NO. 11)(modified tPA signal peptide) MDAMKRGLCCVLLLCGAVFVSPA

(j) (SEQ.ID NO. 12)(Leptin/tPA signal peptide) MHWGTLCCVLLLCGAVFVSPS

(k) (SEQ.ID NO. 13)(Leptin/tPA signal peptide) MHWGTLCCVLLLCGAVFVSP

Relatedly, one may use nucleic acid sequences encoding such signal peptides. The below DNA sequences, with the exception of the modified human leptin signal peptide signal sequence (d, SEQ. ID NO: 6) which was not done, were used as described in the working examples below to encode the corresponding signal peptides, as set forth above.

SEQ. ID NO: 14 (native human leptin signal peptide DNA)
ATGCATTGGGGAACCCTGTGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATG
TCCAAGCT

SEQ. ID NO: 15 (modified human leptin signal peptide DNA)

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ATGCATTGGGGAACCCTGTGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATG
TTTCGCCCAGC

SEQ. ID NO: 16 (modified human leptin signal peptide
DNA)
ATGCATTGGGGAACCCTGTGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATG
TTTCGCCC

SEQ. ID NO: 17 (modified human leptin signal peptide
DNA)
ATGCATTGGGGAACCCTGTGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATG
TTTCGCCCGCT

SEQ. ID NO: 18 (modified human leptin signal peptide
DNA)
ATGCATTGGGGAACCCTGTGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATG
TTTCGAACAGC

SEQ. ID NO: 19 (native human tPA signal peptide DNA)
ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
TCGTTTCGCCCAGC

SEQ. ID NO: 20 (native human tPA signal peptide DNA)
ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
TCGTTTCGCCC

SEQ. ID NO: 21 (modified human tPA signal peptide DNA)
ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
TCGTTTCGAACAGC

SEQ. ID NO: 22 (modified human tPA signal peptide DNA)
ATGGATGCAATGAAGAGAGGGCTCTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCT
TCGTTTCGCCCGCT

SEQ. ID NO: 23 (Leptin/tPA signal peptide DNA)
ATGCATTGGGGAACCCTGTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTT
CGCCCAGC

SEQ. ID NO: 24 (Leptin/tPA signal peptide DNA)
ATGCATTGGGGAACCCTGTGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTT
CGCCC

One may select signal sequences known to be
associated with highly glycosylated proteins. Signal
sequences which may be used are those native to
erythropoietin, Factor VIII, beta-interferon, serum
albumin, insulin, von Willebrand's factor, CD11α, IgG,

follistatin, intrinsic factor, G-CSF, ceruloplasmin,
LAMP-1, secreted hormones, growth factors and other
proteins, human or non-human (such as other primate,
mouse, rat, or other mammal), which are secreted in
5   eukaryotic cells. For yeast cells, yeast α-factor, and
others may be used. Also various other genes have
leader sequences which may facilitate secretion of
proteins in mammalian cells systems, such as human
influenza virus A, human preproinsulin, and bovine
10   growth hormone.

         One may also optimize the amino acid
compositions of the signal sequences on a trial and
error basis to improve glycosylation efficiency, and
prepare non-naturally occurring signal sequences. For
15   example one may increase the number of hydrophobic
amino acid residues or alter the signal peptidase
cleavage site to increase the amount of time the
protein spends in the membrane, in order to prolong the
time period in which the protein is exposed to the
20   cellular "machinery" which accomplishes glycosylation
("machinery" being a shorthand term for those enzymes
and other moieties which perform glycosylation within
the membrane region of the cell).

         It has also been found that substitution of
25   an existing cleavage site (the site at the carboxy-
terminal end of a signal peptide at which the signal
peptide is enzymatically cleaved to generate the mature
protein) with a different cleavage sites may provide
manufacturing advantages, particularly in mammalian
30   cell systems, and increase glycosylation efficiency.
Previously, those skilled in the art had altered enzyme
cleavage sites of prosequences (see below) relating to
signal peptides.

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As will be demonstrated in the working
examples below, use of the tissue plasminogen activator
signal peptide to express a three site glycosylated
leptin protein resulted in a higher glycosylation
5   efficiency than use of the native human leptin signal
peptide. It was further found that the cleavage site
of the tPA (serine-proline-serine) signal peptide, when
substituted into the native human leptin signal
peptide, conferred improved glycosylation efficiency
10   over the use of the non-modified native human leptin
signal peptide.

For particular proteins, the site serine-
asparagine-serine ("SNS") may function to improve the
glycosylation efficiency. For example, as described
15   herein, substitution of the natural human tPA signal
peptide cleavage site with an "SNS" site resulted in a
high yield of correctly cleaved glycosylated leptin
protein (having glycosylation sites at positions 2, 47,
69, and 92). Other cleavage sites include, serine-
20   proline-serine ("SPS"), serine-asparagine-serine
("SNS"), serine-proline ("SP"), and serine-proline-
alanine ("SPA"). A new cleavage site may be
substituted into any signal peptide by known methods,
including site directed mutagenesis of encoding DNA,
25   DNA synthesis, and alteration of genomic DNA within a
cell. One may choose to manufacture a signal peptide,
particularly a signal peptide not found in associated
with any known secreted protein, such as natural signal
peptides, and include a cleavage site as above to
30   optimize or maximize glycosylation efficiency.

Some cleavage sites, such as the serine-
proline-serine cleavage site of natural human tissue
plasminogen activator ("tPA"), are incompletely cleaved

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from the N-terminal region of the mature protein.
Thus, there leaves a serine residue at the N-terminus
of the mature protein, herein referred to as the -1
position.   The present invention also includes the
present leptin glycosylated proteins having, or
optionally having if one chooses to use a subject
cleavage site, one or more amino acid residues at the
N-terminus of the mature protein sequence.

The present invention also includes, more
specifically, glycosylated leptin proteins having
a serine, arginine, proline or alanine
residue at the -1 position,
a serine at the -1 position and a proline at
the -2 position,
a serine-proline-serine sequence at the -1, -
2, and -3 positions,
a serine at the -1 position and an arginine
at the -2 position,
a serine at the -1 position, an arginine at
the -2 position and a serine at the -3 position,
an arginine at the -1 position and a serine
at the -2 position; and,
an alanine at the -1 position and proline at
the -2 positions.

In addition, there may be signal peptide
cleavage sites which cleave a portion of the mature
protein.   Thus, as indicated above, the present
invention includes truncated forms of glycosylated
leptin protein, such as those having up to and
including five amino acid residues deleted from the N-
terminus of the mature protein, such as a leptin
protein of SEQ. ID NO: 1 or 2, having the subject
glycosylation sites.

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Therefore, the present invention also relates
to an improved method of manufacturing a glycosylated
protein comprising:

(a) culturing, under suitable conditions for
5   expression and glycosylation, a host cell containing a
DNA sequence encoding, in the 5' to 3' direction (i) a
signal peptide, and (ii) a DNA encoding a glycosylated
protein; and

(b) obtaining said glycosylated protein
10   wherein said improvement comprises use of a signal
peptide having a peptidase cleavage site optimized for
glycosylation efficiency.

The non-naturally occurring cleavage site may
be selected from among SPS, SP, SNS, and SPA. The
15   signal peptides and glycosylated leptin proteins as set
forth in the specification, including the working
examples, are illustrative, although these methods and
compositions are broadly applicable to a wide variety
of proteins sought to be secreted and/or glycosylated
20   by a eukaryotic cell. Such proteins include but are
not limited to tissue plasminogen activator, Factor
VIII and other blood clotting factors, erythropoietin
and analogs thereof, and other glycosylated proteins.

It was also found that, in conjunction with
25   use of the native leptin leader sequence, use of a
"prosequence" may also improved glycosylation
efficiency. A "prosequence" is an amino acid sequence
optimally having the motif R-X-R/K-R, where "X" is any
amino acid (and the one letter abbreviations are those
30   conventionally used, see infra). The prosequence is
cleavable (after the final R) with furin-like proteases
normally present in CHO cells Watanabe et al., FEBS
letters, 320: 215-218 (1993)(herein incorporated by

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reference). The ability of CHO cells to cleave such
prosequences has been shown to be improved when furin
expression plasmids are transfected into the cells.
Yanagita et al., Endocrinology 133: 639-644
5 (1993)(herein incorporated by reference). For example,
the mature human leptin sequence begins with a valine,
which would interfere with removal of the presequence
by furin. Better prosequence removal could be achieved
by changing this valine to a more preferred amino acid,
10 such as serine or alanine, or inserting such an amino
acid before the valine (by, e.g., site-directed
mutagenesis or other methods available to those skilled
in the art). Therefore, the present methods also
optionally include use of such prosequences in
15 conjunction with the natural leptin signal peptide or
with other signal peptides.

       The present invention also encompasses
compositions, such as nucleic acids, vectors, and host
cells, such as those recited above and herein
20 incorporated by reference, which contain nucleic acids
encoding the present altered signal peptides and/or pro
sequences.

                        EXAMPLES

       The following examples are offered to more
25 fully illustrate the invention, but are not to be
construed as limiting the scope thereof.

       Example 1 demonstrates the Stokes' radius
measurement of various glycosylated leptins.

       Example 2 demonstrates the in vivo biological
30 activity of a one-site glycosylated leptin, denoted
"N48T50". This example demonstrates that this
glycosylated leptin has activity at least equal to
native recombinant human leptin lacking glycosylation.

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Example 3 demonstrates the _in vitro_ biological activity of additional one site glycosylated leptins.

Example 4 demonstrates _in vitro_ biological
5   activity of two-site glycosylated leptin proteins, in terms of a receptor binding assays.

Example 5 demonstrates the effect on glycosylation efficiency of using a threonine residue, rather than a serine residue, in the glycosylation
10 consensus sequence.

Example 6 demonstrates that amino acids adjacent to the consensus sequence affect glycosylation efficiency.

Example 7 that a three site glycosylated leptin
15 protein has a substantially longer systemic circulation time than non-glycosylated leptin.

Example 8 demonstrates in _ob/ob_ mice that a three site glycosylated leptin protein has improved weight loss biological activity as compared to non-
20 glycosylated leptin.

Example 9 demonstrates in _ob/ob_ mice that a three site glycosylated leptin protein has improved appetite suppressant biological activity as compared to non-glycosylated leptin.

25   Example 10 demonstrates in _ob/ob_ mice that intermittent administration of a three site glycosylated leptin has improved weight loss biological activity as compared to non-glycosylated leptin.

Example 11 provides additional dose response
30 studies using a three site glycosylated leptin on wild type animals, demonstrating that a far lower dose of the

three site glycosylated leptin results a substantial
weight loss, as compared to non-glycosylated leptin.

Example 12 provides dose frequency studies
using a three site glycosylated leptin on wild type mice,
5   and demonstrates that a three site glycosylated leptin
may be dosed less frequently than non-glycosylated leptin
to obtain the same weight loss response in animals.

Example 13 sets forth additional multiple
glycosylation site leptin proteins and in vitro
10   biological activity data.

Example 14 sets forth the expression and
glycosylation efficiency of a three site glycosylated
leptin protein using a variety of signal peptides and
other sequences affecting glycosylation or yield.
15   Example 15 sets forth additional expression
data on a variety of multiple glycosylation site leptin
proteins using various signal peptides and other
sequences affecting glycosylation or yield.

Reference Examples of methods used herein
20   follow.

EXAMPLE 1

Stokes' Radius of Various Leptins

25   The present example demonstrates that various
leptins have different Stokes' radii, as determined by
gel filtration. The present example also demonstrates
the consistency of the gel filtration method for
determining the Stokes' radius of a single leptin
30   glycosylated protein, as when repeated measurements were
taken, the measurements varied by less than 2 Å.

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Methods: Gel filtration experiments were
carried out on a Pharmacia FPLC system equipped with a
Unicorn controller for system control, data acquisition
and analysis, a UV-1 detector and a 280 nm filter.
5   Separations were performed at 4°C and at a flow rate of
0.25 ml/min on a SuperDex 200 (HR10/30) column
equilibrated in Dulbeccos phosphate buffered saline.
Protein samples, dissolved in elution buffer, were
applied to the column in 0.25 ml volumes containing 0.1
10 A280 as determined by a Hewlett Packard Model 8435
Spectrophotometer.

The standard proteins found in the Pharmacia
Gel Filtration Calibration Kits, both High Molecular
Weight and Low Molecular Weight, were used as recommended
15 by the manufacturer to calibrate the columns. (As
recommended by the manufacturer, catalase was not used as
a standard.) Additional standards, including human
transferrin (36Å), soybean trypsin inhibitor (22Å) and
horse muscle myoglobin (19Å) were purchased from Sigma
20 Chemicals. Blue Dextran (Pharmacia Gel Filtration
Calibration Kit) was used to define the void volume.
Values for the Stokes' radius (Rs) for any of the various
leptin forms were calculated from a plot of $\sqrt{-\log(Kav)}$
vs Rs where Kav = (Ve-Vo)/Vt-Vo and Ve is the elution
25 volume of the protein, Vo is the void volume, and Vt is
the total bed volume of the column.

Results: Using the above methods, and
SuperDex 200™ as the gel filtration material, the
following Stokes' radii were determined for rHu-Leptin
30 1-146 (SEQ. ID NO: 1, below) having the following
glycosylation sites:

Table 1.1: Stokes' Radius of Various Leptins

rmetHu-leptin 1-146	18.1 Å
rHu-Leptin 1-146 N48T50	23Å
rHu-leptin 1-146 N33T35	24 Å
rHu-leptin 1-146 N47, 69, 102	31.9 Å
rHu-leptin N47, 69, 102	32.3 Å

As can be seen, a population of three site glycosylated leptin protein molecules has a Stokes' radius above 30 Å, as determined by gel filtration. The mean Stokes' radius ((31.9 + 32.3)/2)) is 32.1 Å. The present gel filtration method furthermore demonstrated consistency to one Angstrom. As a comparison, the same glycosylated leptin protein had Stokes' radius of 31.2 Å when determined by sedimentation velocity (using standard methods not detailed here).

The unglycosylated leptin protein (rmetHu-Leptin), as well as the two single site glycosylated leptin proteins, had Stokes' radii less than 30 Å. As will be demonstrated in the working examples below, the N48T50 glycosylated leptin protein had biological activity comparable to rmetHu-Leptin. The three-site glycosylation protein (47, 69, 102) had substantially improved biological activity in terms of increased circulation time (and therefore increase in vivo exposure to drug). This demonstrates the principle that enlarging the effective size (expressed here as the Stokes' radius) prolongs circulation time by decreasing the filterability and ultimate degradation in the kidney.

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EXAMPLE 2

In vivo Biological Activity and Serum Circulation Time of
a One-Site Glycosylated Leptin, N48T50

5   This Example demonstrates that a one-site
glycosylated leptin has biological activity
approximately the same or modestly improved from
rmetHu-Leptin 1-146 (SEQ. ID NO: 1). The glycosylation
did not hinder activity or prevent receptor binding.
10   Further, there is demonstrated that the serum
circulation time of a one-site glycosylated leptin is
the same or modestly longer than rmetHu-Leptin 1-146
(SEQ. ID NO: 1).

          Animals were administered the one-site
15   glycosylated leptin or rmetHu-Leptin at the same dose
daily for 7 days. At the end of 7 days, animals were
sacrificed, and fat content examined. As compared to
rmetHu-Leptin, administration of the one-site
glycosylated leptin resulted in approximately 25%
20   additional fat loss. This demonstrates that the
present glycosylated leptin compositions having a non-
naturally occurring glycosylation site retains
biological activity.
          Methods:
25   1. Leptin Compositions Used. This
glycosylated leptin, "N48T50" had the amino acid
sequence of native human leptin 1-146 (SEQ. ID NO: 1)
with the isoleucine ("I") at position 48 substituted
with asparagine ("N"), and the next two amino acids
30   remaining (leucine ("L") and threonine ("T")) without
substitution. For daily dosing groups (100 ul
injection volume for all): 0.2 mg/ml for 1 mg/kg dose
group, 2.0 mg/ml for 10 mg/kg dose group. For day-0-
only dosing groups: 5 mg/ml concentration, 400 ul
35   injected, 100 mg/kg dose.

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2. Animals:

Number and type. 5 female C57BL/6 mice, from Charles River Laboratories (Wilmington, MA)

Age and weight: Animals were age 8-10 weeks
5   and weighed approximately 20 g each.

3. Administration. At the beginning of each study, mice were weighed and then injected with sample in bolus subcutaneously.

Weighing: Baseline weight was determined in
10   animals allowed to acclimate in animal facility for 1 week prior to study. Baseline weight was taken just before receiving the first dose. Weights were monitored daily throughout each study. After final weights were recorded, animals were sacrificed and the
15   amount of abdominal fat was graded from 0-3, with 0 being no visible fat remaining, and a score of 3 reflecting an amount of visible fat in a normal animal.

Results: Mice treated daily with rmetHu-leptin (1-146) expressed in E.coli lost weight relative
20   to buffer controls as shown in FIGURE 1. Surprisingly the glycosylated leptin (N48 T50 Leptin) treated mice also lost weight. The amount of weight loss increased with increasing dose (1 mg/kg and 10 mg/kg) for both forms of leptin. When single injections of 100 ug/kg
25   were done, the glycosylated leptin treated mice lost more weight than rmetHu-leptin (1-146) treated mice. In addition the weight loss persisted longer for the glycosylated leptin treated mice than for rmetHu-leptin treated mice. Visual examination of rmetHu-leptin and
30   N48 T50 Leptin treated mice indicated that mice treated with both forms of leptin had reduced amounts of abdominal fat and that the amount of abdominal fat was reduced with increased dose. This indicates that the glycosylated leptin is effective at reducing fat

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content and it can be administered less frequently than unglycosylated rmetHu-leptin.

Pharmacokinetic Studies of a One-Site Glycosylated Leptin

This study demonstrates that for intravenous administration, a single-site glycosylated leptin has a longer half life than non-glycosylated leptin. For subcutaneous administration, the circulation times were similar for both the glycosylated and non-glycosylated leptin.

Materials:

1. Leptin. A one site glycosylated leptin as above (sites N48 T50) was used, formulated at 10 mg/ml in Dulbecco's Phosphate Buffered Saline without calcium chloride without magnesium chloride. Recombinant methionyl human leptin 1-146 (SEQ. ID NO: 1 with a methionyl residue at position -1), expressed in E. coli was used as a control, formulated at 2.0 mg/ml in buffer.

2. Animals.

Number used/type: 32 (for glycosylated leptin protein) and 81 (for r-metHu-leptin) male CD-1 mice(Charles River Laboratories, Hollister, CA)

Age/weight: Animals were approximately 6-9 weeks old and weighed approximately 30 grams.

Care/handling: Animals were individually housed and fed a diet of laboratory rodent chow ad libitum. All animals were handled in accordance with good animal handling practices.

3. Administration. Animals were injected with glycosylated leptin at a dose of 1.0 mg/kg body weight intravenously (IV) or subcutaneously (SC).

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4. <u>Sampling</u>. Animals were anesthetized, and
blood samples were collected at designated time points
using standard cardiac puncture techniques. The serum
concentrations of glycosylated leptin were determined
5   using an immunoassay (as described below).

5. <u>Comparison</u>. The circulation time data
were compared to previously obtained data for rmetHu-
Leptin, at the same dose, in similarly sized animals,
using the same routes of administration.

10   <u>Results</u>:

Table 2.1 shows the pharmacokinetic parameters
of glycosylated Leptin and rmetHu-Leptin in mice.
Comparing the IV data, glycosylated Leptin exhibited a
lower systemic clearance (500 mL/h/kg vs. 676 mL/h/kg)
15   and a longer terminal half-life (1.24 h vs. 0.733 h).
The volumes of distribution at steady-state (V_{ss}) were
similar between glycosylated Leptin and rmetHu-Leptin.
These data indicate that the glycosylated protein was
cleared slower than rmetHu-Leptin from the systemic
20   circulation, therefore increasing the half-life and the
exposure (AUC estimates of 2000 ng•h/mL vs. 1480
ng•h/mL). Following the SC dose, similar peak serum
concentrations (C_{max}) were obtained between glycosylated
Leptin and rmetHu-Leptin (1230 ng/mL vs. 1380 ng/mL),
25   although there was a delay in the peak time (t_{max}) for
glycosylated leptin. Similar exposure estimates (based
on AUC) were obtained for both molecules. Subcutaneous
bioavailability was approximately 60.5% for the
glycosylated leptin vs. 79.6% for rmetHu-Leptin.

30

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Table _2.1__

Pharmacokinetic Parameters of Glycosylated
Leptin(N48,T50) and rmetHu-Leptin Following IV and SC
Administration

	Glycosylated Leptin	Leptin	Ratio (Glycosylated Leptin/Leptin)
SC Dose			
t_{max} (h)	0.5	0.167	---
C_{max} (ng/mL)	1230	1380	0.89
AUC (ng•h/mL)	1210	1180	1.03
$t_{1/2, lz}$ (h)	0.552	0.541	0.96
F (%dose)	60.5	79.6	0.760
IV Dose			
AUC (ng•h/mL)	2000	1480	1.35
$t_{1/2, lz}$ (h)	1.24	0.733	1.69
CL (mL/h/kg)	500	676	0.74
V_{ss} (mL/kg)	149	150	0.99

EXAMPLE 3
In vitro biological activity of other one-site
glycosylated leptins

        In Table 3.1, below, the amino acid sequence
location for alteration to include a glycosylation site
is based on the numbering of SEQ. ID NO: 1, above,
which is rHu-Leptin.

        Protein was expressed as in the Reference
Examples below, using the natural human leptin signal
peptide and COS cells. The expression products were
then put through four types of analysis (methods used
are described below):

        1. Expression relative to wild type. The
yield of protein was compared relative to rHu-Leptin
1-146, as expressed in COS cells. The amount of

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rHu-Leptin 1-146 was assigned the number of "1.00" under conditions as defined below.

   2. Percent glycosylation. The yield of fully glycosylated protein was determined as a percent of
5 total leptin protein by visual inspection of a Western Blot, as described below.

   3. Binding, Leptin-R, relative to wild type. In an in vitro competition assay using a preparation of leptin receptor, radio-labeled glycosylated leptin
10 proteins prepared were compared to radio-labeled rHu-Leptin 1-146 in strength of binding to leptin receptor, according to methods described below.

   4. In vitro bioactivity relative to wild type. In an in vitro assay using a chimeric leptin
15 receptor, as described below, glycosylated leptin proteins prepared were compared to rHu-Leptin 1-146, according to methods described below. "ND" means that the data are not available because experiments were not done.
20

Table 3.1

Summary of COS Single Site Glycosylated Leptin expression, binding, and glycosylation results					
Position of N glycosylation 1/	Sequence Changes	Expression Rel. to WT	% Glycosylation	Receptor binding Rel. to WT.	Bioactivity Rel. to WT.
None	wild type	1	0	1	1
4	QKV > NKV	1.7	0	1.7	0.73
5	KVQ > NVT	0.33	65	1.7	0.05
7	QDD > NDT	0.55	5	1.25	0.04
8	DDT > NDT	1.1	15	1.2	1.2
23a	DIS > NIT	7.8	60	ND	0.53
23b	DIS > NIS	ND	ND	ND	ND
25	SHT > NHT	0.13	80	0.4	0.02
26	HTQ > NTT	1.1	70	1.5	0.01
27	TQS > NQT	0.45	30	0.7	0.13
29	SVS > NVT	0.5	70	0.6	0.5
33	KQK > NQT	1.6	95	0.9	0.04
35	KVT > NVT	0.55	95	0.5	0.13

37	TGL > NGT	1.4	95	0.4	0.043
38	GLD > NLT	0.26	45	0.2	0.036
43	PGL > NGT	1.6	85	1.5	0.014
44	GLH > NLT	1.8	10	2	0.78
45	CHP > NHT	0.52	85	0.5	0.08
46	HPI > NPT	1.4	0	0.11	0.27
47	PIL > NIT	1.06	80	0.66	0.84
48	ILT > NLT	0.92	50	0.8	0.53
67	SMP > NMT	1.1	15	0.8	0.52
68	MPS > NAT	0.5	80	0.8	0.036
69	PSR > NST	0.8	75	0.6	1.1
70	SRN > NRT	1.07	10	1	1
71	RNV > NNT	1.84	60	1.9	0.3
72	NVI > NVT	1.4	70	1.7	0.26
73	VIQ > NIT	0.53	45	8	0.01
ESP77*	SND > NNT	0.14	10	2.2	<0.02
92	FSK > NST	4.8	45	ND	0.67
93	SKS > NKT	2.4	5	ND	1.1
97	HLP > NLT	2.6	10	ND	1.1
99	PWA > NWT	0.45	0	0.9	0.5
100a	PWAS > SNAT	0.43	35	0.6	0.9
100b	WAS > NAS	1.2	0	0.7	1.46
100c	WAS > NAT	0.5	20	0.45	0.81
100d	PWAS > ANAT	2.3	35	1.45	0.26
100e	WAS > TAS	1	60 (O-linked)	> 1.9	0.83
101	ASG > NST	0.59	50	0.7	0.33
102	SGL > NGT	0.79	60	0.6	0.85
103	GLE > NLT	1.6	55	1	0.73
115	EAS > NAT	1.1	70	0.9	0.006
116	ASG > NST	1.1	0	0.9	0.56
117	SGY > NGT	1.33	50	1.9	0.01
118	GYS > NYT	1.44	15	0.8	3.8
119	YST > NST	0.61	70	0.8	0.02
120	STE > NTT	0.9	100	0.7	0.01
141	DLS > NLT	0.18	0	0.3	1

* ESP77 indicates a glycosylation site at position 77 and expression using the signal peptide of erythropoietin, as described in more detail infra

1/ "Position" indicates the amino acid position according to SEQ. ID NO: 1, which is rHu-Leptin 1-146. The particular sequence listed (e.g., "53", "55", etc.) indicates the "N" position in the consensus glycosylation sequence of "N - X - S/T".

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Results: As can be seen, with the single-
site glycosylation leptin proteins prepared, compared
to unglycosylated leptin, most single site glycosylated
leptin proteins showed no substantial increase in

5   biological activity as determined by the in vitro assay
used herein, except for the protein with a
glycosylation site at position 118, which appeared to
have an increased amount of activity. Some of these
analogs were secreted at normal or higher levels and

10   most had receptor binding activity comparable to rHu-
Leptin 1-146 expressed and analyzed in the same manner.
Surprisingly some of the glycosylated leptin proteins
had low in vitro biological activity even though they
retained receptor binding activity. Thus the

15   glycosylated leptin proteins could be divided into 2
classes according to whether they retained in vitro
biological activity or not. The glycosylated leptin
proteins that had low in vitro biological activity may
be leptin antagonists.

20

EXAMPLE 4
In vitro biological activity of two-site
glycosylated leptins

25   As presented in Table 4.1, various two-site
glycosylated leptin proteins were also produced and
tested as above for the single site proteins.
Notations and abbreviations are the same as those for
the Table 3.1 for the one-site proteins.

30   The glycosylation notations indicate the
approximate percent of material which had one chain or
two chain, as determined by visual examination of a
Western Blot, as described below. For example, for the
glycosylated leptin protein 25 + 29, 50% of the

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material had one chain, and 5% of the material had two chains.

Table 4.1

Summary of COS Leptin Double Site Expression, Binding, and Glycosylation Results				
Position of N-glycosylation	Expression Rel. to WT	Glycosylation	Receptor binding Rel. to WT.	Bioactivity Rel. to WT.
None	1	0	1	1
25+29	1	1'-50,2'-5	1	ND
25+33	1.2	1'-40,2'-60	1.6	ND
25+35	1.2	1'-70,2'-10	1	ND
26+33	0.46	ND	1.6	ND
26+35	0.59	1'-45,2'-35	1	ND
27+33	0.93	1'-60,2'-30	1	ND
27+35	1.1	1'-50,2'-40	0.7	ND
29+33	1.6	1'-50,2'-45	1.4	ND
29+35	0.33	1'-33,2'-33	0.6	ND
33+48	0.86	1'-35,2'-60	0.6	ND
33+120	0.27	1'-5, 2'-95	0.89	<0.003
35+48	0.46	1'-30,2'-40	0.6	ND
47+69	1.3	1'-10,2'-50	1	0.69
47+102	2.7	1'-50,2'-30	0.86	0.42
48+69	1.63	1'-20,2'-50	0.87	0.81
69+101	1.2	1'-45,2'-20	0.42	0.66
69+102	1.7	1'-40,2'-30	0.5	0.63
69+103	2.6	1'-50,2'-15	1.7	0.67
69+118	2.9	1'-50,2'-5	2.2	2.3
102+100e	1.8	2'-60	0.62	0.97

Many glycosylated leptin proteins containing combinations of two glycosylated sites can be made which retain receptor binding and display biological activity.

Example 5
Improvement of Glycosylation Efficiency
Using a Threonine Rather Than a Serine in the
Consensus Sequence

This example demonstrates that the glycosylation site efficiency is improved using a threonine rather than a serine in the glycosylation

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consensus sequence. In this Example, all amino acid
sequence locations for alterations to include
glycosylation sites are based on the numbering of SEQ.
ID NO: 1, which is rHu-Leptin 1-146.

5   Glycosylated leptin proteins were
constructed, expressed, and analyzed using methods in
the Reference Examples, below. The results are shown in
FIGURE 2. The introduction of a single glycosylation
site by the double substitution W100,S102 to N100,T102
10 resulted in addition of N-linked carbohydrate and the
proportion of molecules containing carbohydrate (by SDS
PAGE as determined by Western blotting) was
substantially more than with a W100,S102 to N100,S102
substitution. This indicates that more of the protein
15 molecules having a threonine in the consensus sequence
were glycosylated than those having a serine in the
consensus sequence. Thus, the glycosylation efficiency
in this expression system is higher using the consensus
sequence Asn-Xxx-Thr than when Asn--Xxx-Ser is used.
20 As such, use of a threonine residue is preferred.

                         EXAMPLE 6
             Glycosylation Efficiency Is Effected
                     by Upstream Sequence
25
          This example shows that glycosylation
efficiency is affected by both the amino acid in the -1
position (relative to the substituted asparagine
residue) as well as substitution of a proline
30 immediately "upstream" (i.e., toward the N-terminus)
from the asparagine residue in the consensus sequence.
          It was found that rHu-Leptin 1-146 with
alterations of: S99, N100, S102 was more efficiently
glycosylated than the same alterations lacking the

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serine substitution at position 99. This indicates
that substitutions around the consensus glycosylation
site can result in additional improvement in
glycosylation site occupancy.

5   In addition, and surprisingly, a W100 to T100
substitution resulted in O-glycosylation of the leptin
presumably at position 100. This indicates that either
O-linked or N-linked carbohydrate can be added to the
same position depending on the particular substitution
10   that is made. FIGURE 2 is a Western Blot comparing the
N-linked glycosylation site to the O-linked
glycosylation site, as indicated. As can be seen, use
of the sequence "TAS" as indicated (with reference to
SEQ. ID NO: 1) results in O-linked glycosylation.
15

EXAMPLE 7
Improved systemic circulation time of a three
site glycosylated leptin

20   This Example demonstrates that a glycosylated
leptin having greater than one glycosylation site has a
circulation time which is substantially longer than
non-glycosylated recombinant human leptin. As can be
seen, glycosylated Leptin exhibited a 4- to 5-fold
25   decrease in systemic clearance and increase in half-life
compared to rmetHu-Leptin. Although there was a small
decrease in subcutaneous bioavailability (~10% decrease
as compared to non-glycosylated), glycosylated leptin
still resulted in a higher drug exposure following
30   subcutaneous dosing.
          Materials:
               1. Leptin. A three site glycosylated leptin
as prepared below (sites 47, 69 and 102, SEQ. ID NO:

32) was used, formulated at 1.76 mg/ml in Dulbecco's
Phosphate Buffered Saline without calcium chloride
without magnesium chloride (Gibco). Recombinant
methionyl human leptin 1-146 (SEQ. ID NO: 1 with a
5   methionyl residue at position -1), expressed in E. coli
was used as a control, formulated at 2.0 mg/ml in
buffer.

  2. Animals.

Number used/type: 27 (for glycosylated leptin protein)
10   and 81 (for r-metHu-leptin) male CD-1 mice(Charles
River Laboratories, Hollister, CA)

Age/weight: Animals were approximately 6-9 weeks old
and weighed approximately 30 grams.

Care/handling: Animals were individually housed and
15   fed a diet of laboratory rodent chow ad libitum. All
animals were handled in accordance with good animal
handling practices.

  3. Administration. Animals were injected
with glycosylated leptin at a dose of 1.0 mg/kg body
20   weight intravenously (IV) or subcutaneously (SC).

  4. Sampling. Animals were anesthetized, and
blood samples were collected at designated time points
using standard cardiac puncture techniques. The serum
concentrations of glycosylated leptin were determined
25   using an immunoassay (as described below).

  5. Comparison. The circulation time data
were compared to previously obtained data for rmetHu-
Leptin, at the same dose, in similarly sized animals,
using the same routes of administration.

30   Results: Results are illustrated in FIGURES 3
and 4. FIGURE 3 is a graph showing the serum leptin
concentration after subcutaneous administration, and

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FIGURE 4 is a graph showing serum leptin concentration after intravenous administration.

In general, following both IV and SC administration of glycosylated leptin having a Stokes' radius greater than about 30 Å, serum concentrations were higher than those observed for rmetHu-Leptin as well as that for a single-site glycosylated leptin (N48 T50). For rmetHu-Leptin, serum concentrations declined below 1.0 ng/mL within 6 hours after both routes of administration; whereas serum concentrations of glycosylated Leptin remained above 1.0 ng/mL for 24 hours after IV or SC administration.

Table 7.1 shows the pharmacokinetic parameters of glycosylated leptin and rmetHu-Leptin in mice.

Table 7.1
Pharmacokinetic Parameters of Glycosylated Leptin and rmetHu-Leptin Following IV and SC Administration

	Glycosylated Leptin	Leptin	Ratio (Glycosylated Leptin/Leptin)
SC Dose			
t_{max} (h)	1	0.167	---
C_{max} (ng/mL)	1430	1380	1.04
AUC (ng•h/mL)	5800	1180	4.93
$t_{1/2, lz}$ (h)	2.21	0.541	4.09
F (% dose)	69.5	79.6	0.873
IV Dose			
AUC (ng•h/mL)	8350	1480	5.65
$t_{1/2, lz}$ (h)	2.76	0.733	3.77
CL (mL/h/kg)	120	676	0.178
V_{ss} (mL/kg)	157	150	1.05

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Comparing the IV data (<u>see</u> FIGURE 4),
glycosylated leptin exhibited a lower systemic clearance
(120 mL/h/kg vs. 676 mL/h/kg) and a longer terminal half-
life (2.76 h vs. 0.733 h). The volumes of distribution

5   at steady-state (V_{ss}) were similar between glycosylated
leptin and rmetHu-Leptin. These data indicate that the
glycosylated protein was cleared slower than
rmetHu-Leptin from the systemic circulation, therefore
increasing the half-life and the exposure (AUC estimates

10   of 8350 ng•h/mL vs. 1480 ng•h/mL). Following the SC
dose, (<u>see</u> FIGURE 3) similar peak serum concentrations
(C_{max}) were obtained between glycosylated leptin and
rmetHu-Leptin (1430 ng/mL vs. 1380 ng/mL), although there
was a delay in the peak time (t_{max}) for glycosylated

15   leptin. Similar to the results obtained from IV
administration, subcutaneously administered glycosylated
leptin exhibited an increased terminal half-life (2.21 h
vs. 0.541 h) and area under the curve ("AUC") (5800
ng•h/mL vs. 1180 ng•h/mL), probably due to the decreased

20   systemic clearance for glycosylated leptin. Subcutaneous
bioavailability was approximately 69.5% for the
glycosylated leptin vs. 79.6% for rmetHu-Leptin.

EXAMPLE 8
25   Improved Weight Loss Activity of Three-Site
Glycosylated Leptin

This Example demonstrates that a glycosylated
human leptin having a Stokes' radius greater than 30 Å
30   has improved <u>in</u> <u>vivo</u> biological activity as compared to
non-glycosylated recombinant human leptin. As can be
seen, with daily administration after 7 days, *ob/ob* mice
lost ~6.8 times more weight with the three-site

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glycosylated leptin here administered than with
non-glycosylated leptin.

Methods:

1. Leptin. The three-glycosylation site
5 leptin, prepared as described below (sites 47, 69 and
   102, SEQ. ID NO: 32), was used, formulated at a
   concentration of 1.9 mg/ml in Dulbecco's phosphate-
   buffered saline, pH 6.8. Recombinant methionyl human
   leptin 1-146 (SEQ. ID NO: 1 with a methionyl at position
10 -1) was used as a basis for comparison, formulated at 20
   mg/ml in 10 mM sodium acetate with 5% sorbitol, pH 4.0.
   Ten mM sodium acetate with 5% sorbitol, pH 4.0, was used
   as a vehicle control.

2. Animals. Animals were housed in a
15 temperature-, light-, and humidity-controlled conditions,
   with lights on at 0600 hours and lights off at 1800
   hours. The Amgen, Inc. animal research facility is
   approved by the USDA and AAALAC-accredited. Six female
   ob/ob mice (Jackson Laboratories) were used per treatment
20 group. Mice were 2 months old at the time of study, and
   weighed an average of 45.6 grams. Mice were randomized
   to treatment groups such that the mean body weights of
   the groups were equivalent prior to treatment initiation.
   Animals were housed two per cage and fed standard
25 laboratory rodent chow pellets ad libitum.

3. Administration. All treatment procedures
   were approved by Amgen's Institutional Animal Care And
   Use Committee. Glycosylated leptin, r-metHu-Leptin, or
   placebo were administered daily via subcutaneous
30 injection in the mid-scapular region in a volume of 0.1
   ml. The dose of leptin was 0.5 mg/kg body weight/day,
   for both glycosylated leptin and r-metHu-Leptin.

Injections were given on 7 consecutive days, beginning on study day 0, in the late afternoon (within 2 hours of lights off in the colony). Animals were weighed daily at the time of injection. All data are reported as the mean

5   ± SE.

Results:

     As can be seen in FIGURE 5, the three-site glycosylated leptin ("GE-Leptin") resulted in the largest amount of weight loss, with an average weight loss of

10   10.8 ± 0.3 grams (-23.8 ± 0.5 % of initial body weight). Administration of the same dose of r-metHu-Leptin ("hLeptin") produced an average weight loss of 1.6 ± 0.4 grams (-3.5 ± 1.1 % of initial body weight), while administration of placebo resulted in an average weight

15   gain of 2.6 ± 0.2 grams (5.7 ± 0.3 % of initial body weight). This Example demonstrates a substantially improved *in* *vivo* biological activity of glycosylated leptin as compared to non-glycosylated recombinant human leptin.

20

EXAMPLE 9
Improved Appetite Suppressant Activity of Three-Site
Glycosylated Leptin

25   This Example demonstrates that a glycosylated human leptin having a Stokes' radius greater than 30 Å has improved *in* *vivo* biological activity as compared to non-glycosylated recombinant human leptin. As can be seen, with daily administration after 7 days, *ob/ob* mice

30   ate ~11 times less food with the glycosylated leptin than with non-glycosylated leptin.

     Methods:

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1. <u>Leptin</u>. The three-glycosylation site leptin, prepared as described below (sites 47, 69 and 102, SEQ. ID NO: 32), was used, formulated at a concentration of 1.9 mg/ml in Dulbecco's phosphate-
5   buffered saline, pH 6.8. Recombinant methionyl human leptin 1-146 (SEQ. ID NO: 1 with a methionyl at position -1) was used as a basis for comparison, formulated at 20 mg/ml in 10 mM sodium acetate with 5% sorbitol, pH 4.0. Ten mM sodium acetate with 5% sorbitol, pH 4.0, was used
10 as a vehicle control.

2. <u>Animals</u>. Animals were housed in a temperature-, light-, and humidity-controlled conditions, with lights on at 0600 hours and lights off at 1800 hours. The Amgen, Inc. animal research facility is
15 approved by the USDA and AAALAC-accredited. Six female <i>ob/ob</i> mice (Jackson Laboratories) were used per treatment group. Mice were 2 months old at the time of study, and weighed an average of 45.6 grams. Mice were randomized to treatment groups such that the mean body weights of the
20 groups were equivalent prior to treatment initiation. Animals were housed two per cage and fed standard laboratory rodent chow pellets <u>ad libitum</u>.

3. <u>Administration</u>. All treatment procedures were approved by Amgen's Institutional Animal Care And
25 Use Committee. Glycosylated leptin, r-metHu-Leptin, or placebo were administered daily via subcutaneous injection in the mid-scapular region in a volume of 0.1 ml. The dose of leptin was 0.5 mg/kg body weight/day, for both glycosylated leptin and r-metHu-Leptin.
30 Injections were given on 7 consecutive days, beginning on study day 0, in the late afternoon (within 2 hours of lights off in the colony.

4. <u>Food Measurement</u>. Food intake was measured daily at the time of injection by weighing the amount of food in each animal's cage each day. Food intake is reported as the grams eaten per mouse per day, and was

5 calculated as follows: (weight of the food in the cage the previous day - weight of the food that day)/the number of mice per cage (two). All data are reported as the mean ± SE.

<u>Results</u>:

10 As can be seen in FIGURE 6, administration of the three-site glycosylated leptin resulted in the largest reduction of food intake, with an average food intake of 0.4 ± 0.04 grams/mouse/day for the final 24-hour period after the seventh dose. Administration of

15 recombinant methionyl human leptin produced a reduction in food intake to 4.4 ± 0.4 grams/mouse/day, in comparison to the food intake of vehicle-treated controls (7.0 ± 0.3 grams/mouse/day), for the same 24-hour period. This Example demonstrates a substantially improved <u>in</u>

20 <u>vivo</u> biological activity of glycosylated leptin as compared to non-glycosylated recombinant human leptin.

EXAMPLE 10

Improvement in Weight Loss Activity of Three-
25 Site Glycosylated Leptin Administered Intermittently

This Example demonstrates that the improved <u>in</u> <u>vivo</u> biological activity of a three site glycosylated human leptin having a Stokes' radius greater than 30 Å is

30 maintained when the material is administered on an intermittent basis. As can be seen, *ob/ob* mice lost significantly more weight with either daily or every-other-day administration of glycosylated leptin, than

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when treated with a 10-fold higher dose of non-glycosylated leptin.

Methods:

　　1. Leptin. The three-glycosylation site
5　leptin, prepared as described below (sites 47, 69 and
102, SEQ. ID NO: 32), was used, formulated at a
concentration of 1.9 mg/ml in Dulbecco's phosphate-buffered saline, pH 6.8. Recombinant methionyl human
leptin 1-146 (SEQ. ID NO: 1 with a methionyl at position
10　-1) was used as a basis for comparison, formulated at 20
mg/ml in 10 mM sodium acetate with 5% sorbitol, pH 4.0.
Ten mM sodium acetate with 5% sorbitol, pH 4.0, was used
as a vehicle control.

　　2. Animals. Animals were housed under
15　temperature-, light-, and humidity-controlled conditions,
with lights on at 0600 hours and lights off at 1800
hours. The Amgen, Inc. animal research facility is
approved by the USDA and AAALAC-accredited. Six female
ob/ob mice (Jackson Laboratories) were used per treatment
20　group. Mice were 4.5 months old at the time of study,
and weighed an average of 66.6 grams. Mice were
randomized to treatment groups such that the mean body
weights of the groups were equivalent prior to treatment
initiation. Animals were housed two per cage and fed
25　standard laboratory rodent chow pellets ad libitum.

　　3. Administration. All treatment procedures
were approved by Amgen's Institutional Animal Care And
Use Committee. Glycosylated leptin, r-metHu-Leptin, or
placebo were administered either daily or every-other-day
30　via subcutaneous injection in the mid-scapular region in
a volume of 0.1 ml. The dose of leptin was 0.25 or 2.5
mg/kg body weight/day for mice injected daily with

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glycosylated leptin or r-metHu-Leptin. The dose of
leptin was 1 or 10 mg/kg body weight/day for mice
injected every-other-day with glycosylated leptin or r-
metHu-Leptin. Injections were given on 7 consecutive
5   days, beginning on study day 0, in the late afternoon
(within 2 hours of lights off in the colony). Mice
injected every other day with leptin received injections
of vehicle on alternate days. Animals were weighed daily
at the time of injection. Percent weight loss is
10   calculated as: ((Body weight on day 7 - Body weight on
day 0)/Body weight on day 0) multiplied by 100. All data
are reported as the mean ± SE.

       Results:

              As shown in Table 10.1, mice injected daily
15   with 0.25 mg/kg body weight/day glycosylated leptin lost
more weight than did mice receiving either the same dose
or a ten-times higher dose of recombinant methionyl human
leptin.

              Table 10.1: Weight loss (expressed as a % of
20   initial body weight) after 7 days of daily dosing of
Glycosylated Leptin or recombinant methionyl human
leptin.

	Dose	
Injectate	0.25 mg/kg/d	2.5 mg/kg/d
r-metHu-Leptin	-8.2 ± 1.0%	-15.2 ± 0.5%
Three-Site Glycosylated Leptin	-21.4 ± 0.6%	not done

              As shown in Table 10.2, mice injected every-
other-day with 1 mg/kg body weight/day glycosylated
25   leptin lost more weight than did mice receiving either

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the same dose or a ten-fold higher dose of recombinant
methionyl human leptin.

Table 10.2: Weight loss (expressed as a % of initial
body weight) after 7 days of daily dosing of glycosylated
5 leptin or recombinant methionyl human leptin.

	Dose	
Injectate	1.0 mg/kg/d	10 mg/kg/d
r-metHu-Leptin	-5.7 ± 0.8%	-10.6 ± 0.8%
Three-Site Glycosylated Leptin	-16.9 ± 1.0%	not done

This example demonstrates that the enhanced
biological activity of glycosylated leptin, relative to
non-glycosylated leptin, is preserved when the protein is
administered intermittently to obese mice.

10

EXAMPLE 11
Dose Response Studies of a Three-Site Glycosylated
Leptin on Wild Type Mice

15   This example demonstrates that the present
three-site glycosylated leptin having a Stokes' radius
greater than 30 Å has biological activity in non-obese
mice. Moreover, the present example confirms in wild type
mice, that a far lower dose of the glycosylated leptin
20 results in substantial weight loss, compared to non-
glycosylated leptin

        1. Leptin. The three-glycosylation site
leptin, prepared as described below (sites 47, 69 and
102, SEQ. ID NO: 32), was used, formulated at a
25 concentration of 5.1 mg/ml in Dulbecco's phosphate-
buffered saline, pH 6.8. Recombinant methionyl human
leptin 1-146 (SEQ. ID NO: 1 with a methionyl at position

-1) was used as a basis for comparison, formulated at 20
mg/ml in 10 mM sodium acetate with 5% sorbitol, pH 4.0.
Ten mM sodium acetate with 5% sorbitol, pH 4.0, was used
as a vehicle control.

5   2. Animals. Animals were housed under
temperature-, light-, and humidity-controlled conditions,
with lights on at 0600 hours and lights off at 1800
hours. The Amgen, Inc. animal research facility is
approved by the USDA and AAALAC-accredited. Six female
10 C57Bl/6J mice (Jackson Laboratories) were used per
treatment group. Mice were 2.5 months old at the time of
study, and weighed an average of 20.0 grams. Mice were
randomized to treatment groups such that the mean body
weights of the groups were equivalent prior to treatment
15 initiation. Animals were housed two per cage and fed
standard laboratory rodent chow pellets ad libitum.

          3. Administration. All treatment procedures
were approved by Amgen's Institutional Animal Care And
Use Committee. Glycosylated leptin, r-metHu-Leptin, or
20 placebo were administered either daily via subcutaneous
injection in the mid-scapular region in a volume of 0.1
ml. The dose of leptin was 1 or 10 mg/kg body
weight/day. Injections were given on 7 consecutive days,
beginning on study day 0, in the late afternoon (within 2
25 hours of lights off in the colony. Animals were weighed
daily at the time of injection. Percent weight loss is
calculated as: ((Body weight on day 7 - Body weight on
day 0)/Body weight on day 0) multiplied by 100. All data
are reported as the mean ± SE.

30   Results:

          As shown in Table 11.1, mice injected daily
with 1 mg/kg body weight/day glycosylated leptin lost

more weight than did mice receiving either the same dose
or a ten-times higher dose of recombinant methionyl human
leptin.

5   Table 11.1: Weight loss (expressed as a % of
initial body weight) after 7 days of daily dosing of
Glycosylated Leptin or recombinant methionyl human
leptin.

	Dose	
Injectate	1 mg/kg/d	10 mg/kg/d
r-metHu-Leptin	-2.2 ± 1.0%	-3.9 ± 0.6%
Three-Site Glycosylated Leptin	-8.6 ± 0.6%	not done

This example demonstrates that the enhanced
biological activity of glycosylated leptin, relative to
10   non-glycosylated leptin, is also present in non-obese
mice.

EXAMPLE 12

Improvement in Weight Loss Activity of Three-Site
15   Glycosylated Leptin Administered Intermittently to Wild
Type Mice

The present example demonstrates that the
three-site glycosylated leptin having a Stokes' radius
20   greater than 30 Å has a improved biological activity as
compared to r-metHu-Leptin 1-146. Further, the example
demonstrates that the improved biological activity is
sustained when glycosylated leptin is administered on a
less frequent dosing schedule than r-metHu-Leptin 1-146,
25   in wild type mice.

1.   Leptin. The three-glycosylation site
leptin, prepared as described below (sites 47, 69 and

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102, SEQ. ID NO: 32), was used, formulated at a
concentration of 5.1 mg/ml in Dulbecco's phosphate-
buffered saline, pH 6.8. Recombinant methionyl human
leptin 1-146 (SEQ. ID NO: 1 with a methionyl at position
5   -1) was used as a basis for comparison, formulated at 20
mg/ml in 10 mM sodium acetate with 5% sorbitol, pH 4.0.
Ten mM sodium acetate with 5% sorbitol, pH 4.0, was used
as a vehicle control.

        2. Animals. Animals were housed under
10   temperature-, light-, and humidity-controlled conditions,
with lights on at 0600 hours and lights off at 1800
hours. The Amgen, Inc. animal research facility is
approved by the USDA and AAALAC-accredited. Six female
C57B1/6J mice (Jackson Laboratories) were used per
15   treatment group. Mice were 2.5 months old at the time of
study, and weighed an average of 20.0 grams. Mice were
randomized to treatment groups such that the mean body
weights of the groups were equivalent prior to treatment
initiation. Animals were housed two per cage and fed
20   standard laboratory rodent chow pellets ad libitum.

        3. Administration. All treatment procedures
were approved by Amgen's Institutional Animal Care And
Use Committee. Glycosylated leptin, r-metHu-Leptin, or
placebo were administered either daily via subcutaneous
25   injection in the mid-scapular region in a volume of 0.1
ml. The dose of leptin was 1, 5, or 10 mg/kg body weight,
injected every-other-day. Leptins were injected every-
other-day for 7 consecutive days, beginning on study day
0, in the late afternoon (within 2 hours of lights off in
30   the colony). Mice received injections of vehicle on
alternate days. Animals were weighed daily at the time
of injection. Percent weight loss is calculated as:
((Body weight on day 7 - Body weight on day 0)/Body

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weight on day 0) multiplied by 100. All data are
reported as the mean ± SE.

Results:

As shown in Table 12.1, mice injected every-
5   other-day with 1, 5, or 10 mg/kg body weight glycosylated
leptin lost more weight than did mice receiving the same
of recombinant methionyl human leptin every-other-day.

Table 12.1: Weight loss (expressed as a % of
initial body weight) after 7 days of every-other-day
10 dosing of glycosylated leptin or recombinant methionyl
human leptin.

	Dose		
Injectate	1 mg/kg	5 mg/kg	10 mg/kg
r-metHu-Leptin	0.8 ± 1.4%	-1.0 ± 0.8%	-1.5 ± 0.9%
Three-Site Glycosylated Leptin	-1.2 ± 2.1%	-6.7 ± 1.2%	-9.1 ± 0.5%

This example demonstrates that the enhanced
biological activity of glycosylated leptin, relative to
non-glycosylated leptin, is preserved when the protein
15 is administered intermittently to non-obese mice.

EXAMPLE 13
Additional Multiple Glycosylation Site Leptin Proteins

20   Presented below in Table 13.1 are additional
glycosylated human leptin proteins which were also
prepared. The table columns present: (1) the position
of the N-glycosylation (with respect to the numbering
of SEQ. ID NO 1., rHu-Leptin 1-146)(unless otherwise
25 noted); (2) the expression yield as compared to "wild
type" ("WT", here, rHu-Leptin 1-146 as in SEQ. ID NO:
1); (3) the glycosylation species which were detected;
(4) the receptor binding relative to "WT"; (5) the

bioactivity relative to "WT". Methods used are described below. The term "ND" means not determined. (The specific sequences used herein for expression are more fully set forth in the Example below pertaining to expression of glycosylated leptin protein 47 + 69 + 102)

Table 13.1

Summary of COS Leptin Three Site Glycosylation expression, binding, and glycosylation results				
Position of N-glycosylation	Expression Rel. to WT	Glycosylation	Receptor binding Rel. to WT.	Bioactivity Rel. to WT.
None	1	0	1	1
2+69+92	1.07	1'-25,2'-45,3'-10	0.7	0.41
2+69+92RRR*	0.36	1'-10,2'-25,3'-60	ND	ND
26+33+48	0.25	1'-30,2'-30,3'-20	ND	ND
26+35+48	0.88	1'-30,2'-30,3'-20	1.3	ND
26+33+115	0.17	1'-30,2'-30,3'-20	ND	ND
26+35+115	0.16	1'-30,2'-30,3'-20	ND	ND
27+35+115	0.25	1'-30,2'-30,3'-20	0.8	ND
29+33+115	0.83	1'-30,2'-30,3'-20	1.1	ND
33+48+115	0.27	1'-25,2'-25,3'-40	0.7	ND
35+48+115	0.47	1'-30,2'-30,3'-20	1	ND
47+69+100e	2.66	1'-5,2'-80(50/O-link)	0.66	1.3
47+69+102	1.8	1'-30,2'-50,3'-10	0.62	0.86
47+69+103	0.57	1'-20,2'-45,3'-5	0.44	0.11
48+69+101	2.2	1'-10,2'-60,3'-10	0.95	0.15
48+69+102	3.3	1'-20,2'-45,3'-20	1.04	0.46
48+69+103	2.8	1'-15,2'-60,3'-5	0.52	0.36
48+69+118	1.2	1'-20,2'-45,3'-5	0.09	1.6
69+102+118	1.1	1'-50,2'-30,3'-0	0.11	3.5
69+103+118	0.72	1'-50,2'-20,3'-0	0.55	1.5

Abbreviations and notations are the same as those used above, see, e.g., Table 4.1.

* "RRR" denotes the use of three C-terminal arginine residues on the glycosylated leptin protein.

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Additionally, the following multiple site glycosylated leptin proteins have been made and tested as further described in Example 15, below.

2+23+47+69+92

2+47+69+92+102

23+47+69+92+102

2+47+69+92

2+47+69+102

23+47+69+102

23+47+69+92

Q-47+69+92+102 ("Q-" indicates that SEQ. ID NO: 2, rHu-Leptin 1-145, was used as the protein backbone to which glycosylation sites were added)

47+69+100e+102

5

In addition, the present invention also encompasses a glycosylated leptin protein having glycosylation sits at position 47, 69, 92, and 102.

10   EXAMPLE 14
Expression of a Three Site Glycosylated Leptin Protein
Using a Variety of Signal Sequences and Other Sequences
Affecting Glycosylation

15   This example illustrates the differences in glycosylation of a three-site glycosylated leptin protein having sites available for glycosylation located at positions 47, 69, and 102 of rHu-Leptin 1-146 (SEQ. ID NO: 1 having the noted glycosylation sites
20   using the formula N-X-S/T, prepared as described herein), also as described above. Expression in two

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cell types, COS cells, and CHO cells, was generally according to methods in the Reference Examples, below. Glycosylation was determined according to the methods set forth in the Reference Examples, below. The degree

5   of glycosylation was scored on a scale of 1 to 5, with 5 having the appearance of the maximum occupancy of glycosylation sites. The term "ND" means "not determined."

A variety of signal sequences were used. The

10   amino acid sequences of these signal sequences are presented below in Table 14.1. The following terms were used to denote what signal sequences or other amino acid sequences used to express the present glycosylated leptin:

Leptin - the naturally occurring human leptin signal sequence
Leptin/TPA(L/T) - the first five n-terminal of human leptin followed by 15 human tPA signal amino acids ending with SP
TPA/Leptin(T/L) - the seven N-terminal amino acids from human tissue plasminogen activator followed by 16 amino acids of the human leptin signal sequence beginning at LCG
Leptin(SP)- the naturally occurring human leptin signal sequence, except having the last two c-terminal amino acid replaced with serine-proline
Leptin(SPS)- the naturally occurring human leptin signal sequence, except having the two c-terminal amino acids replaced with serine-proline-serine
Leptin(SNS)-the naturally occurring leptin signal sequence from human leptin, except having the two c-terminal amino acids replaced with serine-asparagine-serine
Leptin-pro- the naturally occurring human leptin signal sequence, plus an additional "pro" sequence at the c-terminus
Leptin-modified(LGDVMT)- the naturally occurring human leptin signal sequence, except with six c-terminal amino acids substituted with LGDVMT

Leptin + RRR @ c-term - the naturally occurring human leptin signal sequence, and additionally, at the c-terminus of the glycosylated leptin protein, three arginine residues
Leptin R81, R85(analog)- the naturally occurring human leptin signal sequence, and glycosylated leptin protein being the amino acid sequence of SEQ. ID NO: 1 with arginine at positions 81 and 85, and glycosylation sites at positions 47, 69 and 102
Thrombopoietin (TPO)- the naturally occurring human thrombopoietin signal sequence
Tissue Plasminogen Activator (TPA)- the naturally occurring human tissue plasminogen activator signal sequence
TPA(SNS)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids serine-asparagine-serine
TPA(SPA)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids serine-proline-alanine
TPA(SP)- the naturally occurring human tissue plasminogen activator signal sequence having the two c-terminal amino acids being the amino acids serine-proline
TPA(SFS)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids serine-phenylalanine-serine
TPA(SWS)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids serine-tryptophan-serine
TPA(INS)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids isoleucine-asparagine-serine
TPA(INA)- the naturally occurring human tissue plasminogen activator signal sequence having the three c-terminal amino acids being the amino acids isoleucine-asparagine-alanine
TPA-A2- the naturally occurring human tissue plasminogen activator signal sequence having additional c-terminal amino acids of arginine-glycine-arginine-phenylalanine-arginine-arginine
TPA(SP)-A2 the naturally occurring human tissue plasminogen activator signal sequence having the last serine of the naturally occurring sequence eliminated and

having additional c-terminal amino acids of arginine-glycine-arginine-phenylalanine-arginine-arginine
TPA-A4- the naturally occurring human tissue plasminogen activator signal sequence additional c-terminal amino acids of glutamine-glutamic acid-isoleucine-arginine-glycine-arginine-phenylalanine-arginine-arginine
TPA-A5- the naturally occurring human tissue plasminogen activator signal sequence having additional c-terminal amino acids of glutamine-glutamic acid-isoleucine-histidine-alanine-arginine-phenylalanine-arginine-arginine
Intrinsic factor- the naturally occurring human intrinsic factor signal sequence
Serum albumin(pre-pro)- the naturally occurring human serum albumin signal sequence and prosequence
G-CSF- the naturally occurring human granulocyte colony stimulating factor signal sequence
von Willebrand's factor (vW)- the naturally occurring human von Willebrand's factor signal sequence
MAC-1 (CD11 alpha)- the naturally occurring human CD11α signal peptide
Tie (receptor)- the naturally occurring human Tie receptor signal sequence
Factor VIII - the naturally occurring human Factor VIII signal sequence
IgG-1, murine - the naturally occurring murine IgG-1 signal sequence
Follistatin (FS)- the naturally occurring human follistatin signal sequence
LAMP-1 - the naturally occurring human LAMP-1 signal peptide
Ceruloplasmin (CP)- the naturally occurring human ceruloplasmin signal peptide
EPO (or "ESP" denoting erythropoietin signal peptide)- the naturally occurring human erythropoietin signal sequence
EPO(ESP)RRR@c-term - the naturally occurring human erythropoietin signal sequence and also having, at the c-terminus of the amino acid sequence for the glycosylated leptin protein, three arginine residues
EPO-HSApro - the naturally occurring human erythropoietin signal sequence having at the c-terminus the "pro" sequence from human serum albumin
EPO-modifiedHSApro - the naturally occurring human erythropoietin signal sequence having at the c-terminus a modified "pro" sequence from human serum albumin (modified as indicated in the table below)

EPO-modifiedHSApro +furin - the naturally occurring human erythropoietin signal sequence having at the c-terminus a modified "pro" sequence from human serum albumin (modified as indicated in the table below) coexpressed with furin
EPO-NT3 pro- the naturally occurring human erythropoietin signal sequence having at the c-terminus a "pro" sequence from NT-3
EPO-HSApro (leptin NH2 VtoA)- the naturally occurring human erythropoietin signal sequence having at the c-terminus a "pro" sequence from human serum albumin and with Val1 of the leptin sequence changed to Ala to improve cleavage of the prosequence by furin.

Table 14.1

Expression of Leptin 47+69+102 Glycosylated Protein

Signal	Sequence of leader peptide	Glycosylation 1-5, 5-Best	
		COS	CHO
Leptin	MHWGTLCGFLWLWPY LFYVQA	1	1
Leptin/TPA(L/T)	MHWGT/LCCVLLLCG AVFVSP	4	5
TPA/Leptin(T/L)	MDAMKRG/LCGFLWL WPYLFYVQA	1	0.5
Leptin(SP)	MHWGTLCGFLWLWPY LFYVSP	4	3
Leptin(SPS)	MHWGTLCGFLWLWPY LFYVSPS	4.5	4.5
Leptin(SNS)	MHWGTLCGFLWLWPY LFYVSNS	ND	ND
Leptin-pro	MHWGTLCGFLWLWPY LFYVQA-RGRFRR	3	4.5
Leptin-modified(LGDVMT)	MHWGTLCGFLWLWPY LGDVMT	3	2

Leptin + RRR @ c-term	MHWGTLCGFLWLWPY LFYVQA	5	5
Leptin R81, R85(analog)	MHWGTLCGFLWLWPY LFYVQA	0.5	ND
Thrombopoietin (TPO):	MELTELLLVV MLLLTARLTL S	1	1
Tissue Plasminogen Activator (TPA)	MDAMKRGLCCVLLLC GAVFVSPS	4.5	4.5
TPA(SNS):	MDAMKRGLCCVLLLC GAVFVSNS	4	4
TPA(SPA)	MDAMKRGLCCVLLLC GAVFVSPA	4.5	4
TPA(SP)	MDAMKRGLCCVLLLC GAVFVSP	3	3
TPA(SFS)	MDAMKRGLCCVLLLC GAVFVSFS	3.5	4
TPA(SWS)	MDAMKRGLCCVLLLC GAVFVSWS	3.5	4
TPA(INS)	MDAMKRGLCCVLLLC GAVFVINS	3.5	4
TPA(INA)	MDAMKRGLCCVLLLC GAVFVINA	3.5	3
TPA-A2	MDAMKRGLCCVLLLC GAVFVSPS-RGRFRR	5	4.5
TPA(SP)-A2	MDAMKRGLCCVLLLC GAVFVSP-RGRFRR	5	5
TPA-A4	MDAMKRGLCCVLLLC GAVFVSPS- QEIRGRFRR	4.5	4
TPA-A5	MDAMKRGLCCVLLLC GAVFVSPS- QEIHARFRR	4.5	4
Intrinsic factor	MAWFALYLLS LLWATAGT	3	no expression

Serum albumin(pre-pro)	MKWVTFISLLFLFSS AYSRG-RGVFRR	1	ND
G-CSF	MAGPATQSPMKLMAL QLLLWHSALWTVQEA	1	1
von Willebrand's factor (vW)	MIPARFAGVLLALAL ILPGTLC	1.5	3
MAC-1 (CD11 alpha)	MALRVLLLTALTLCH G	2	2
Tie (receptor)	MVWRVPPFLLPILFL ASHVGA	1	1
Factor VIII	MQIELSTCFFLCLLR FCFS	1	1
IgG-1, murine	MKCSWVIFFLMAVVT GVDS	1	3
Follistatin (FS)	MVRARHQPGG LCLLLLLLCQ FMEDRSAQA	1	1
LAMP-1	MAPRSARRPL LLLLPVAAAR PHALSSA	low expression	no expression
Ceruloplasmin (CP)	MKILILGIFL FLCSTPAWA	1	3
EPO (ESP):	MGVHECPAWL WLLLSLLSLP LGLPVLG	3	1.5
EPO(ESP)RRR@c-term	MGVHECPAWL WLLLSLLSLP LGLPVLG	5	5
EPO-HSApro	MGVHECPAWLWLLLS LLSLPLGLPVLG- RGVFRR	4	4.5
EPO-modifiedHSApro	MGVHECPAWLWLLLS LLSLPLGLPVLG- RGRFRR	4	4.5
EPO-modifiedHSApro +furin	MGVHECPAWLWLLLS LLSLPLGLPVLG- RGRFRR	4	4.5

EPO-NT3 pro	MGVHECPAWLWLLLS LLSLPLGLPVLG-NRTSRRKR	3.5	3
EPO-HSApro (leptin NH2 VtoA)	MGVHECPAWLWLLLS LLSLPLGLPVLG-RGVFRR	4	4

The various TPA signal peptides, particularly those with modified c-termini (such as the addition of a prosequence) appeared to have the greatest
5   glycosylation efficiency in both CHO and COS cells.
         This was confirmed in a Western Blot (FIGURE 7). As can be seen, the use of the signal peptide for tPA resulted in the highest molecular weight, and therefore the most highly glycosylated, sample.
10   FIGURE 8 is a Western blot which shows the results of a comparison of the various expression conditions for the three-site glycosylated leptin protein 47 + 69 + 102 as above. Beginning at the left hand side of the Western Blot in the FIGURE, the lanes
15   are loaded as follows:
     Lane 1: molecular weight standards;
     Lane 2: 47+69+102 having a c-terminal amino acid sequence of three arginines, expressed in COS cells using the native human leptin signal peptide;
20   Lane 3: Same as lane 2, expressed in CHO cells
     Lane 4: Same as lane 2, expressed using a native human erythropoietin signal peptide;
     Lane 5:   "QTT COS ESP", rHu-Leptin 1-145 (SEQ. ID NO: 2) having the amino acid change 27T29S > 27T29T,
25   expressed in COS cells using a native human erythropoietin signal peptide;
     Lane 6: "QTT CHO ESP", same as lane 5, expressed in CHO cells using a native human erythropoietin signal peptide;

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Lane 7: "EA2 47 + 69 + 102 COS", same as Lane 2,
lacking the C-terminal arginines, expressed in COS
cells, using the erythropoietin signal peptide and a
modified human albumin prosequence, as indicated in
5   this Example;

Lane 8: "EA2 47 + 69 + 102 CHO", same as lane 7,
expressed in CHO cells;

Lane 9: "EA2 47 + 69 + 102 + Furin in CHO" same as lane
8, using the furin construct as indicated in this
10   Example.

As can be seen by noting the density of high
molecular weight bands (indicating glycosylation), the
triple glycosylated leptin protein (lanes 2, 3, 4, 7,
8, an 9) has more glycosylation than the modified rHu-
15   Leptin 1-145, having a single O-linked site (lanes 5
and 6). Use of CHO cells resulted in an increased
amount of glycosylation as compared to COS cells (lane
2 versus lane 4, for example), and the use of the
erythropoietin signal peptide appeared also to improve
20   glycosylation (lanes 3 versus lane 5, for example).

FIGURE 9 is a Western Blot comparing the use
of either the leptin signal peptide or the tPA signal
peptide, or use of either one with a substituted enzyme
cleavage site of the other. Use of the tPA signal
25   peptide resulted in greater glycosylation than use of
the leptin signal peptide (left two lanes). When the
c-terminal portion of the leptin signal peptide
containing its peptidase cleavage site was used with
the N-terminal portion of the tPA signal peptide,
30   glycosylation efficiency decreased ("Tpa/Leptin"
lanes). But when the C-terminal portion of the tPA
signal peptide containing its cleavage site was used
with the N-terminal portion of the leptin signal

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peptide, glycosylation efficiency increased ("Leptin
/Tpa" lanes). Good glycosylation efficiency was found
when only the tPA cleavage site was introduced into the
leptin signal peptide("Leptin(SPS)"). It had greater
5   glycosylation efficiency than substitution of a partial
cleavage site sequence("Leptin(SP)").

FIGURE 10 is a Western Blot comparing
glycosylation efficiency by observation of results of
removal of the carbohydrate moiety by N-glycanase. As
10 can be seen, in the lanes which have carbohydrate
removed (indicated by the "+"), the leptin protein
migrates to the same molecular weight as non-
glycosylated leptin. Comparing the apparent amount of
carbohydrate in the lanes without N-glycanase ("-"),
15 use of the erythropoietin signal peptide ("ESP" or "E"
in combination with another notation, as used above)
appears to more efficiently glycosylated the three site
glycosylated leptin protein tested.

20   EXAMPLE 15
              Additional Expression Studies of Multiple site
          Glycosylated Leptin Proteins Using a Variety of Signal
             Peptides and Other Sequences Affecting Glycosylation

25   This example presents additional data of
expression of single and multiple glycosylation site
leptin proteins, using a variety of signal peptides and
other sequence. Unless otherwise indicated, the
glycosylated leptin proteins below refer to
30 glycosylation sites added to SEQ. ID NO: 1, using the
preferred formula of N-X-S/T. Expression was in COS
cells.

           The percent("%") glycosylation means that
percent of the molecules containing any carbohydrate.
35 This was determined by visual examination of a Western

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Blot (as further described below in the Reference
Examples)and determining subjectively that proportion
of glycosylated protein of total leptin protein
visualized.

5   Controls

        Presented below in Table 15.1 are the data
for various leptin proteins. The human leptin 1-145
(SEQ. ID NO: 2, herein denoted to be "Q-") was
expressed as a glycosylated protein. When expressed in
10   COS cells using its native glycosylation site, using a
signal peptide from erythropoietin ("ESP" as in the
Examples above), there was 25% glycosylation
(indicating that 25% of the available sites for
glycosylation were glycosylated in a population of
15   expressed protein molecules). This was above the 10%
glycosylation seen when the native human leptin signal
peptide (as above) was used. When one of the
glycosylation sites was changed to have a threonine at
position 29 rather than a serine (as shown in the
20   Table), the results were doubled, indicating that a "T"
is better than an "S" for O-glycosylation. Both the "T"
and the "S" of the natural O-linked site may each be
glycosylated with a mixture of one and two carbohydrate
chains. When the site is changed to have a "T" at
25   position 29, the percent of these two sites having 1
and/or two chains is increased.

Table 15.1:

Position of glycosylation	Sequence Change	Expression Rel. to WT	% Glycosylation	Receptor binding Rel. to WT.	Bio activity Rel. to WT.
WT	none	1	0	1	1
ESP Ob+	EPO sp	2	0	1	0.95
(-Q) Ob+	(-)Q @ 28	1.3	10	1.3	0.14
ESP (-Q) Ob+	EPO sp	1.1	25	0.6	0.36
ESP (-Q[TT])Ob+	27T29S > 27T29T	0.72	60	0.71	0.3
EA Ob+		0.13	0	3.5	1.8

5   "WT" denotes rHu-Leptin 1-146 (SEQ. ID NO: 1)

"ESP" denotes the native human erythropoietin signal

peptide, as set forth in Example 14, above

"ESP Ob+" denotes use the above with the native human

erythropoietin signal peptide

10 "(-Q)Ob+" denotes rHu-Leptin 1-145 (SEQ. ID NO:2)

"EA" denotes use of the native human erythropoietin

signal peptide with the human albumin prosequence, as

in Example 14, above.

Expression of Single Site Glycosylated Leptin Proteins

15

Site 2 Comparisons

        As can be seen from Table 15.2, below, the

addition of three terminal arginines resulted in

20 improved glycosylation efficiency.

Table 15.2

Position of glycosylation	Expression Rel. to WT	% Glycosylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
ESP2	0.3	60	0.9	0.16, 0.012
EA V>A2	0.45	35	1, 0.7	0.53, 0.73
2RRR	ND	90	ND	ND

Where two assays were done, both results are presented.
The abbreviations are the same as those for Example
14,above. "ND" as used in all Tables, means not
25 determined.

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Site 23 comparisons

As indicated in Table 15.3, the highest
expression levels and the highest bioactivity occurred
5   with use of the native leptin signal peptide (first
row, 23a). Expression was in COS cells.

Table 15.3

Position of glycosylation	Sequence Changes	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
23a	DIS > NIT	7.8	50	ND	0.53
ESP* 23a	EPO sp	0.49	30	0.5	0.79
ESP (-Q) 23	EPO sp	0.78	45	0.87	<0.03
ESP (-Q[TT]) 23	EPO sp	0.32	60	ND	<0.004

The abbreviations are the same as those for Example
14,above. "ND" as used in all Tables, means not
10 determined.

Site 47 Comparisons

As can be seen in Table 15.4, below, the
addition of three terminal arginine residues ("RRR")
15 resulted in additional glycosylation for the
glycosylated leptin protein with a glycosylation site
at position 47.

Table 15.4

Position of glycosylation	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
47	1.06	80	0.66	0.84
47RRR	0.07	95	ND	0.86

Where two assays were done, both results are presented.
20 The abbreviations are the same as those for Example
14,above. "ND" as used in all Tables, means not
determined.

Site 48 Comparisons

25 As presented in Table 15.5, below, the
highest level of expression for the position 48 single

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site glycosylated leptin protein using COS Cells was
with the signal peptide from erythropoietin combined
with the prosequence from human serum albumin. As can
be seen, this single site protein had a biological

5   activity higher than non-glycosylated leptin.

Table 15.5

Position of N-glycosylation	Sequence Changes	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
48	ILT > NLT	0.92	50	0.8	0.53
ESP 48	EPO sp	0.54	75	0.8	<0.001
EA 48	EPO sp + HSA pro	1.8	80	1	1.2
AA 48	HSA sp + HSA pro	1	90	1	0.78

The abbreviations used are the same as those used for
the above Examples. Percent glycosylation is expressed
in the same terms as Table 15.1, above.

10   Site 69 Comparisons

As can be seen from Table 15.6, use of the
tPA signal peptide plus three terminal arginines
resulted in the highest glycosylation efficiency.

Table 15.6

Position of N-glycosylation	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
69	0.8	75	0.6	1.1
T 69	ND	85	ND	ND
69RRR	0.07	65	ND	ND
T 69RRR	0.04	95	ND	ND

15   The abbreviations used are the same as those used for
the above Examples. Percent glycosylation is expressed
in the same terms as Table 15.1, above.

Site 92 Comparisons

20   As can be seen in Table 15.7, the addition of
three terminal arginines improved glycosylation
efficiency.

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Table 15.7

Position of N-glycosylation	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
92	4.8	45	1.6	0.8
92RRR	0.03	95	ND	ND

The abbreviations used are the same as those used for the above Examples. Percent glycosylation is expressed in the same terms as Table 15.1, above.

Site 102 Comparisons

As can be seen in Table 15.8, the addition of three C-terminal arginine residues resulted in improved glycosylation efficiency.

Table 15.8

Position of N-glycosylation	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
102	1.8	70	0.5	0.66
102RRR	0.07	95	ND	0.33

The abbreviations used are the same as those used for the above Examples. Percent glycosylation is expressed in the same terms as Table 15.1, above.

Expression of Two Site Glycosylated Leptin Protein
Site 47+69 Comparisons

As can be seen in Table 15.9, for site 47 + 69 leptin glycosylated protein (as described above), use of the native leptin signal peptide gave the highest expression levels. Use of the erythropoietin signal peptide with the human serum albumin prosequence, or use of the human serum albumin signal peptide and prosequence, gave higher bioactivity results.

Table 15.9

Position of N-glycosylation	Sequence Changes	Expression Rel. to WT	Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
47+69	47+69	1.3	1'-10,2'-50	1	0.69
ESP 47+69	EPO sp	0.35	1'-10,2'-60	0.6	<0.002
EA 47+69	EPO sp + HSA pro	0.3	1'-5,2'-85	0.3	1.8
AA 47+69	HSA sp + HSA pro	0.82	1'-20,2'-50	1	1.7

Abbreviations are the same a those used above.
Glycosylation is expressed in the same terms as used
above, see, e.g., Table 4.1.

5   Site 69+102 Comparisons

        As can be seen in Table 15.10, use of the
erythropoietin signal peptide in COS Cells apparently
had a detrimental effect on expression of the 69 + 102
two site glycosylated leptin (as described above).

10
Table 15.10

Position of N-glycosylation	Sequence Changes	Expression Rel. to WT	% Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
69+102	69+102	1.7	1'-40,2'-30	0.5	0.63
ESP 69+102	EPOsp /69+102	0.6	1'-20,2'-50	1	<0.001
47+102	47+102	2.7	1'-50,2'-25	1.08	0.42

Abbreviations are the same as those used above, see,
e.g., Example 14 for more details. Glycosylation is
expressed in the same terms as used above, see, e.g.,
15   Table 4.1.

Expression of Three Site Glycosylated Leptin Protein

        As can be seen in Table 15.11, the use of the
erythropoietin signal peptide had varying effects on
various glycosylated leptin proteins expressed in COS
20   cells. Interestingly, the glycosylated leptin protein
with the highest bioactivity had the highest receptor
binding (the lower the number the higher the affinity
for the receptor).

Table 15.11

Position of glycosylation	Sequence Changes	Expression Rel. to WT	Glyco-sylation	Receptor binding Rel. to WT.	Bio-activity Rel. to WT.
ESP 2+47+69	EPO sp	0.2	1'-5,2'-70,3'-20	0.7	0.24
L/T 2+47+69	(seeEx14)	0.21	1'-5,2'-60,3'-30	1.25	ND
L(SNS) 2+47+69	(seeEx14)	0.31	ND	1.63	ND
T 2+47+69	(seeEx14)	0.1	1'-5,2'-50,3'-40	ND	ND
ESP 23+47+69	EPO sp	0.51	1'-5,2'-10,3'-45	0.5	1.4
ESP 47+69+77	EPO sp	0.51	1'-10,2'-75,3'-5	4.2	<0.006
ESP 47+69+92	EPO sp	0.39	1'-15,2'-50,3'-15	0.71	0.47
ESP (-Q) 47+69+92	EPO sp	0.76	1'-15,2'-50,3'-15	0.8	0.88

Abbreviations are the same as those used above, see,
e.g., Example 14 for more details. Glycosylation is
5   expressed in the same terms as used above, see, e.g.,
Table 4.1.

Expression of Four Site Glycosylated Leptin Protein

         As can be seen in Table 15.12, for quadruple
10 site glycosylated leptin protein expression in COS
cells, various expression levels were obtained, and the
resultant glycosylated proteins had various degrees of
receptor binding and bioactivity. For this group of
quadruple site leptins, the 23 +47 + 69 + 92 and 23 +
15 47 + 69 + 102 site leptins had the highest bioactivity
relative to wild type (i.e., rmetHu-Leptin 1-146, SEQ.
ID NO: 1).

Table 15.12

Summary of COS Leptin Glycosylation Quadruple Site Expression, Binding, and Glycosylation Results				
Position of glycosylation	Expression Rel. to WT	Glycosylation	Receptor binding Rel. to WT.	Bioactivity Rel. to WT.
None	1	0	1	1
ESP2+47+69+92RRR	0.004	1'-5,2'-5,3'-30,4'-70	ND	ND
2+47+69+92RRR	0.13	1'-5,2'-5,3'-60,4'-25	ND	0.2
T 2+47+69+92	0.052	1'-5,2'-5,3'-45,4'-40	ND	ND
T 2+47+69+92RRR	0.01	1'-52'-5,3'-35,4'-50	ND	ND
T(SNS) 2+47+69+92	0.15	1'-5,2'-20,3'-45,4'-25	1.1	<0.1
T(-S)2+47+69+92	0.14	1'-5,2'-20,3'-45,4'-25	1.1	<0.01
EA2 2+47+69+92	0.24	1'-5,2'-10,3'-50,4'-30	ND	ND
TA4 2+47+69+92	0.24	1'-10,2'-25,3'-15,4'-45	0.6	0.49
TA5 2+47+69+92	0.13	1'-10,2'-25,3'-15,4'-45	0.8	0.88
L/T 2+47+69+92	0.2	1'-5,2'-25,3'-45,4'-25	1.2	ND
L(SNS) 2+47+69+92	0.28	ND	0.75	ND
T 2+47+69+102	0.16	ND	ND	ND
L(SNS) 2+47+69+102	0.28	ND	1.1	ND
ESP 23+47+69+102	0.48	1'-20,2'-20,3'-20,4'-10	0.5	1.8
ESP 23+47+69+92	0.41	1'-20,2'-20,3'-20,4'-5	0.5	2.3
ESP(-Q) 47+69+92+102	0.32	1'-10,2'-40,3'-40	0.57	0.13
47+69+100e+102	1.5	1'-25,2'-40,3'-20	0.7	0.66

Abbreviations are the same as those used above, see,
e.g., Example 14 for more details. Glycosylation is
expressed in the same terms as used above, see, e.g.,
Table 4.1.

Expression of Five Site Glycosylated Leptin Protein

As presented in Table 15.13, the expression
levels of various quintuple site glycosylated proteins
was fairly low using the indicated signal peptides.
Some data were not determined ("ND").

Table 15.13

Summary of COS Leptin Glycosylation Quintuple Site Expression, Binding, and Glycosylation Results				
Position of N-glycosylation	Expression Rel. to WT	Glycosylation	Receptor binding Rel. to WT.	Bioactivity Rel. to WT.
None	1	0	1	1
2+23+47+69+92 RRR	0.11	1'-5,2'-5,3'-20,4'-40,5'-25	1	ND
T 2+23+47+69+92	0.045	2'-5,3'-20,4'-40,5'-25	ND	ND
T 2+23+47+69+92RRR	0.01	2'-5,3'-10,4'-45,5'-30	ND	ND
T 2+47+69+92+102	0.19	2'-20,3'-30,4'-30,5'-15	.83	ND
ESP 23+47+69+92+102	0.34	1'-20,2'-20,3'-20,4-20,5'-5	0.3	1.3
L/T 2+47+69+92+102	0.29	2'-30,3'-30,4'-30,5'-5	0.75	ND
L(SPS)2+47+69+92+102	0.18	1'-5,2'-10,3'-20,4-30,5'-25	1.42	ND
T(SNS)2+47+69+92+102	0.16	2'-30,3'-30,4-30,5'-5	0.5	ND
T(SPA)2+47+69+92+102	0.19	2'-20,3'-30,4-30,5'-15	0.58	ND
L(SNS)2+47+69+92+102	0.21	ND	0.38	ND

Abbreviations are the same as those used above, see, e.g., Example 14 for more details. Glycosylation is expressed in the same terms as used above, see, e.g., Table 4.1.

In addition, Western Blots of the present glycoslated leptin proteins also illustrates that differences in expression conditions and compositions results in different glycosylation efficiencies. FIGURE 11 shows that increasing the number of glycosylation sites, at least up to five sites, increases the amount of glycosylation found on the leptin protein when expressed in CHO cells. The samples are as follows:

Lane 0: "MOCK" is non-leptin containing cell culture supernatant;

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Lane 1:   "ESP(W.T.)", rHu-Leptin 1-146 (SEQ. ID NO: 1)
expressed using an erythropoietin signal peptide;
Lane 2:   "ESP (N48)", same as above, using the single
site glycosylated leptin protein indicated (as
5   described above);
Lane 3:   "ESP (N47 + N69)", same as above, using the
two-site glycosylated leptin protein indicated (as
described above);
Lane 4:   "ESP (N47 + N69 + N102)", same as above, using
10   the three site glycosylated leptin protein indicated
(as described above);
Lane 5:   "Tpa(SNS) N2 + N46 + N69 + N92" indicates the
four site glycosylated leptin protein as described
above, expressed using a human tPA signal peptide
15   having an enzyme cleavage site of SNS (see Example 14
for sequence information);
Lane 6:   "Tpa N2 + N23 + N47 + N69 + N92" indicates the
five site glycosylated leptin protein as described
above, expressed using the natural human tPA signal
20   peptide (see Example 14 for sequence information).
        As can be seen, the molecular weight
increases with the increase in glycosylation sites
(compare lane 1 to lane 6). This indicates that the
sites are adding carbohydrate and that up to five
25   chains can be added to leptin simultaneously.

N-terminal amino acids/peptides
        The below Table 15.14 presents a comparison
of the different N-terminal amino acids of the subject
30   glycosylated leptin protein incident to the use of
various substituted enzyme cleavage sites for various
signal peptides.

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Table 15.14

Signal Peptide	Glycosylation	Amino Terminus N-terminal extension
Leptin	+	Correct (V)
TPA	++++	S+ SPS
TPASP	++	30% SP
TPA(SNS)	+++	96%
Leptin(SPS)	++++	S
Leptin(SP)	+++	Not Determined
Leptin/tPA	+++	S

As can be seen, the most highly glycosylated leptins having the highest yield of correct N-terminal amino acid was produced using the signal peptide tPA(SNS). Leptin(SNS) also was highly glycosylated, and produced a leptin having an N-terminal amino acid of serine (e.g., serine at the -1 position of SEQ. ID NO: 1 in a glycosylated leptin with a modified amino acid sequence of SEQ. ID NO: 1).

REFERENCE EXAMPLES

The following reference examples provide methods which were used in the above Working Examples. Preparation of DNAs, Vectors and Host Cells

1. Construction of the Human Leptin (1-146) expressing vector. These methods result in an expression vector for rHu-Leptin 1-146 (SEQ. ID NO: 1) in mammalian cells. The DNA encoding rHu-Leptin 1-146 including its signal peptide was also used as a template for preparing glycosylated leptin proteins of the present invention.

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A DNA encoding rHu-Leptin amino acids 1-146
plus a signal sequence as in Zhang et al., Nature 372:
425-432 (1994) at 430, Figure 6b, herein incorporated
by reference in its entirety, was cloned from human
5   adipose cDNA by polymerase chain reaction (PCR). The
signal sequence cloned encoded the following amino acid
sequence:

     M H W G T L C G F L   W L W P Y L F Y V Q   A

     Primers. The 5' (forward) flanking primer
10   encoded the amino terminus of the rHu-Leptin signal
peptide, an XbaI restriction enzyme site, and an
optimized Kozak sequence (TCT ATC TAG ACC ACC ATG CAT
TGG GGA ACC CTG T). The 3' (reverse) flanking primer
sequence (GAG AGT CGA CTA TCA GCA CCC AGG GCT GA)
15   contained the complement of the carboxyl terminus of
rHu-Leptin(1-146) and termination codons, as well as a
SalI restriction site.

     Vector Preparation. The PCR amplification
product was digested with XbaI and SalI restriction
20   enzymes, electrophoresed on an agarose gel, then
isolated from the gel using the Promega® Wizard® kit
procedure (Promega® Corporation, Madison, WI). The
purified product was ligated to XbaI and SalI cut
pDSRα2 expression vector modified slightly from that
25   described in WO 90/14363 1990, e.g., at Figure 12,
herein incorporated by reference in its entirety. The
pDSRα2 used herein maintained the same functional
elements, but was slightly modified from that set forth
in WO 90/14363. The sequence at the Hind III site was
30   modified to AAGCTTCTAGA to generate an XbaI site, and
the sequence at the NcoI site was modified to
GTCGACCTAGG to generate a SalI site, with sufficient
DNA sequence ("stuffer DNA") in between the two sites
to allow efficient cutting by both XbaI and SalI to
35   produce the cut plasmid for directional cloning of the

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present leptin protein expression construct. The
resulting pDSRα2/leptin plasmid was used for mammalian
cell expression as below, and as a template for _in
vitro_ site directed mutagenesis.

5   2.   Construction of Glycosylation Sites into
Leptin by site-directed mutagenesis. Glycosylation
sites were introduced into rHu-Leptin 1-146 (SEQ. ID
NO: 1, above) sequence by site-directed mutagenesis
using overlap extension PCR methods similar to those
10   described by Ho et al., Gene Vol.77, pp. 51-59 (1989).
The pDSRa2 Leptin plasmid, prepared as above, was
utilized as a PCR template for the initial steps of
site directed mutagenesis.

         PCR. PCR procedures were performed in two
15   successive steps.

         Step 1: Two reactions (PCR1 and PCR2) were
performed on leptin template DNA using a total of four
oligonucleotides: the 5' (forward) flanking rHu-Leptin
primer, a reverse mutagenic primer, a forward mutagenic
20   primer (complementary at least in part to the reverse
mutagenic primer) and the 3' (reverse) flanking rHu-
Leptin primer. The mutagenic primers contained the
desired nucleotide changes as well as 6-20 nucleotides
exactly matching the template on each side of the
25   changes. PCR1 used the 5' (forward) flanking primer
and the reverse mutagenic primer. PCR2 used the 3'
(reverse) flanking primer and the forward mutagenic
primer. The DNA products of PCR1 and PCR2 contained
overlapping sequences at and on both sides of the
30   mutations. The amplified DNA fragments were separated
by agarose gel electrophoresis. Small pieces of
agarose containing DNA fragments of the correct size
were excised from the gel.

         Step 2: The DNA fragments from PCR1 and PCR2
35   were combined together and a third PCR reaction was

performed using only the 5' forward and 3' reverse
flanking primers. Annealing of the complementary 3'-
terminal regions of the appropriate strands of the PCR1
and PCR2 products and subsequent strand elongation
5   resulted in formation of full-length leptin DNA
fragments. Thus, a full length DNA segment containing
the desired mutations was amplified.

PCR for expression in COS and CHO cells. For
expression in the human embryonic kidney cell line 293
10   (such as that commercially available from the American
Type Culture Collection) the 5' (forward) primer
contained sequences which introduced a stop codon, a
KpnI site and a Kozak sequence (ACCACC) in front of the
leptin signal peptide coding region
15   (5'-TCTGGTACCTAGACCACCATGCATTGGGGAACCCTGT-3'). The
3'(reverse) primer
(5'-GAAGCGGCCGCCTATCAGCACCCAGGGCTGA-3') contained
sequences which introduced two stop codons (TGA TAG)
and a NotI restriction site at the end of the
20   glycosylated leptin protein coding region. For COS and
CHO cell expression the 3' (reverse) primer contained
sequences that introduced a stop codon followed by a
SalI restriction site (GAGAGTCGACTATCAGCACCCAGGGCTGA).
The 5' forward reaction primer
25   (TCTATCTAGACCACCATGCATTGGGGAACCCTGT) had an XbaI
restriction site followed by a Kozak sequence upstream
of the leptin initiator codon (ATG).

PCR Methods. Polymerase chain reactions were
performed using either of two procedures
30   interchangeably.

In one method, used in some of the
constructions for 293 expression, PCR reactions were
performed using a protocol adapted from Cheng et. al.,
PNAS 91: 5695 (1994)(herein incorporated by reference
35   in its entirety): 4 µl each of forward and reverse

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primers (5 pm/µl), 1 µl template (50 ng), 10 µl of 5X
LP buffer (100 mM Tricine, pH 8.7/25% glycerol/425 mM
KOAc), 2 µl dNTP stock (1 mM each of dATP, dTTP, dCTP,
dGTP), 0.8 µl rtTh polymerase (Perkin Elmer®; 2.5

5   U/µl), and 2 µl Vent polymerase (NEB; 0.01 U/µl after
1:100 fresh dilution in 1X LP buffer). H2O was added
to bring the final volume to 50 µl. All the components
were added together in the order shown and the PCR was
started when the temperature during the first cycle was

10   above 60°C by adding 1 µl of 10 mM MgOAc. Reaction
conditions were: 2 cycles of 94°C, 10 sec/45°C, 1 min/
68°C, 5 min followed by 25 cycles of 94°C, 10 sec/55°C,
1 min/ 68°C, 5 min. The amplified fragments were
separated by agarose gel electrophoresis and the

15   correct sized DNA fragment was purified using a
Geneclean™ kit and procedures supplied by the
manufacturer (Bio 101, Inc.) herein incorporated by
reference. The purified DNA was digested with NotI and
KpnI, then it was purified again using the Geneclean™

20   kit. Digestion conditions were 20 Units KpnI in "M"
buffer (22 µL final volume) (Boehringer Mannheim)
followed by addition of 3 µl of "H" buffer, 20 units
NotI in 52 µl final volume. The fragment was then
ligated into plasmid pBCB cut with KpnI and NotI.

25   Plasmid pBCB was derived from pRC/CMV (Invitrogen®,
Carlsbad, California) by deletion of the region of
pRC/CMV comprising the f1 origin, SV40 origin, neomycin
resistance gene and SV40 polyadenylation site. Ligated
DNA was precipitated with 2 volumes of ethanol in 0.3M

30   NaOAc pH 5.2 in the presence of carrier tRNA and
transformed into E. coli. Glycosylated leptin protein
DNAs were initially screened by colony PCR to identify
clones containing the correctly sized and type of DNA
insert. With this procedure, cells containing plasmids

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were placed into PCR tubes in the presence of leptin
forward and reverse primers. The mixture was then
subjected to PCR using the reaction conditions
described above. Plasmids from positive clones were
5   then prepared and the glycosylated leptin protein
insert was sequenced to confirm the presence of the
desired glycosylation sites and to ensure that no
additional amino acid changes were introduced.

A slightly different PCR strategy was used in
10   the remainder of the constructions. PCR was performed
using Taq DNA Polymerase (Boehringer Mannheim) or
preferably, the proofreading DNA polymerase Pfu
polymerase (Stratagene), which unlike Taq polymerase
does not tend to add an extra untemplated nucleotide at
15   the 3' terminus of the extended strands. In Taq
polymerase PCRs the DNA template was combined with 2.5
μl 10X Taq PCR buffer, 2.5 μl 1mM dNTPs, 5 pmol of each
primer, and water in a final volume of 25 μl. 0.5 units
of Taq polymerase was added after the PCR mixture
20   reached 94°C. PCR reactions were then carried out for
25 cycles at 94°C for 30 seconds, 60°C for 30 seconds,
and 72°C for 30 seconds. In Pfu polymerase PCRs, the
DNA template was combined with 5 ul 10xPfu
buffer(Stratagene), 5 μl 1mM dNTPs, 10 pmol of each
25   primer, and water in a final volume of 50 μl and 1.2 U
Pfu polymerase were added. Four cycles of PCR with an
annealing temperature of 48°C were performed followed
by 20 cycles with an annealing temperature of 66°C. In
each cycle denaturation was performed for 30 sec at
30   94°C, annealing was performed for 30 sec, and
elongation was at 74°C for 30 sec. Following the two
PCR reactions of the first step described earlier,
product bands of the correct sizes were excised from an
agarose gel following electrophoresis and the gel
35   slices containing the products of PCR 1 and PCR 2 were

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either added directly to a PCR tube for the second step
of PCR, or first combined in a tube with 300 µl of
water and boiled to melt the agarose before adding to
the PCR tube. PCRs were performed with Taq or Pfu

5   polymerase as described earlier using the forward and
reverse flanking primers. After the final cycle of
PCR, the tubes were allowed to incubate an extra 5
minutes at the elongation temperature. The resulting
PCR products for each analog were cleaned using the

10   Promega® Wizard® PCR Cleanup kit. The purified DNA
was digested in a 50 ul restriction digest with XbaI
and SalI restriction enzymes (Boehringer Mannheim) at
37°C for 1 hour. The digests were cleaned by Promega®
Wizard® Cleanup kit. The digested fragment was then

15   ligated into XbaI and SalI digested pDSRa2 vector. A 1
µl aliquot of the ligation reaction, containing pDSRa2
Leptin analog plasmid, was used to transform DH10B
cells by electroporation. A single colony for each
analog was grown overnight in liquid culture and

20   plasmid was isolated using the Qiagen® Maxi DNA®
plasmid isolation kit. DNA for each pDSRa2 Human
Leptin analog was resuspended in water and sequenced to
ensure that the correct sequence was present.
        Multiple glycosylation sites. Two or more

25   glycosylation site mutations were combined by
introducing a new substitution into DNA already
containing a change, using the same PCR process. To
construct double glycosylation site leptin analog
genes, single site glycosylated leptin plasmids

30   (produced as described above) were used as PCR
templates, and an additional glycosylation site was
introduced by site directed mutagenesis with the
appropriate primers. Similarly, plasmids encoding
leptin analogs with three glycosylation sites were

constructed using double N-glycosylation site leptin
DNAs as template, and the process could be iterated for
introduction of further glycosylation sites.
Alternatively, new combinations of glycosylation site

5   mutations can be produced by using two different DNA
templates, containing different glycosylation sites, in
PCR1 and PCR2. For example, a DNA encoding an analog
with glycosylation sites at positions 2, 47, 69 and 102
could be produced by the mutagenic primer pair for

10   position 47 glycosylation, by using a DNA template with
a glycosylation site at position 2 in PCR1 and a DNA
with glycosylation sites at positions 69 and 102 in
PCR2.

These general procedures were used to

15   construct plasmids for expression of the glycosylated
leptin proteins shown in the Examples above The DNA
sequence changes for each of the forms are shown.

Chimeric signal peptides. Constructs for
expression of leptin or a leptin analog with a non-

20   leptin signal peptide were prepared by overlap
extension PCR methods for gene splicing (Horton et al.,
Gene 77: 61-68 (1989)) similar to those used for site-
directed mutagenesis. In a preliminary step, a DNA
encoding the signal peptide of the exogenous gene, for

25   example tissue plasminogen activator (TPA), was
obtained by cloning methods or by a combination of
chemical and enzymatic gene synthesis. This DNA was
used as template in a PCR to generate a DNA fragment
encoding the exogenous signal peptide preceded by a

30   consensus Kozak sequence and immediately followed by
the first part of the coding region of mature rHu-
leptin (or leptin analog). The primers used in this
PCR reaction were a 5' (forward) flanking primer for

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the exogenous gene and a reverse primer whose 5'
portion (10-25 nucleotides) was complementary to the
sequence encoding the amino terminal portion of mature
leptin (or leptin analog). A second PCR reaction was
5 performed with leptin or leptin analog DNA as template,
using the leptin 3'(reverse) flanking primer and a
forward primer encoding the region of the junction of
the exogenous signal peptide and the mature leptin (or
leptin analog) sequence. The forward primer in this
10 reaction was designed to overlap the DNA generated by
the first PCR, usually over a length of 15-35
nucleotides. The products of the two PCR reactions
were gel purified as described earlier, mixed, and were
annealed and amplified in a PCR with just the exogenous
15 gene 5'(forward) flanking primer and the 3' (reverse)
flanking leptin primer.

Host Cells

1. <u>Expression of Glycosylated Leptin
Proteins in 293 cells</u>. DNA was transfected into the
20 human embryonic kidney cell line, "293", (such as that
commercially available from the American Type Culture
Collection) using the lipofectamine method. 293 cells
were grown to 40-70% confluency in tissue culture
plates(P100) in 293 medium (DMEM(Difco®)/20mM HEPES/1X
25 Pen-Strep-Glutamine/20% FBS). 20 µg of plasmid DNA
encoding the glycosylated leptin protein in 1 ml of
DMEM was filter sterilized with a 0.45 µm Acrodisc®
membrane (Gelman Sciences). 100 µL of lipofectamine
(Gibco®/BRL®) was added and the DNA-lipofectamine mix
30 was incubated for 20 minutes at RT. Medium was removed
from plates containing 293 cells and 4 mL of DMEM and
the DNA/lipofectamine solution was added. After 4-6

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hours at 37°C, 5 ml of DMEM with 20% fetal bovine serum
was added and the cultures incubated overnight. The
next day the cells were rinsed with 293 medium and 5 ml
of fresh 293 medium was added. The conditioned medium
5   was collected after 3 days, aliquoted and stored at
-70°C.

2. Expression of glycosylated leptin
proteins in COS cells. cDNA clones of glycosylated
leptin proteins were transferred into COS-1 cells (ATCC
10   No. CRL-1650) by electroporation. COS-1 cells were
harvested from semi-confluent dishes, washed with
medium (DMEM containing 10% fetal bovine serum and 1%
L-glutamine/penicillin/ streptomycin (Irvine
Scientific)) and resuspended at 6 x 10^6 cells/ml. One
15   half ml of cells was transferred to a 0.2 cm
electroporation cuvette (Bio-Rad®) and electroporated
with a BTX Electroporation System Electrocell
Manipulator 600® at 650 uF and 130 volts on the low
voltage setting with 25 µg of plasmid DNA encoding the
20   glycosylated leptin protein. The electroporated cells
were plated on 100 mm tissue culture dish in 7 ml of
medium. The conditioned medium was collected 3 days
after electroporation, filtered using a 0.45 µm
Acrodisc® membrane (Gelman Sciences)and stored at
25   minus 80°C.

3. Expression of glycosylated leptin protein
in CHO cells. Stable expression of rHu-Leptin 1-146 or
glycosylated leptin protein was performed by
transformation of dihydrofolate reductase deficient
30   (DHFR⁻) Chinese Hamster Ovary (CHO) cells with pDSRα2
with the selected glycosylated leptin protein DNA as
above followed by isolation and testing of individual

clones. A 60 mm tissue culture dish was plated with

$1x10^6$ CHO DHFR$^-$ cells grown in CHO D$^-$ medium (DMEM-high

glucose, 10% fetal bovine serum, 1% penicillin/

streptomycin/glutamine, 1% nonessential amino

5   acids(Gibco®) and 1% HT supplement (Gibco®)) the day

before transfection. A 10 µg DNA precipitate was then

formed and added to the plates dropwise as per the

Mammalian Cell Transfection Kit instructions (Specialty

Media, incorporated herein by reference). After 24

10   hours in a tissue culture incubator, the medium was

replaced with fresh CHO D- medium. Twenty four hours

later the cells were split into six 100 mm culture

dishes with CHO select medium (D-MEM high glucose, 10%

dialyzed fetal bovine serum, 1% penicillin

15   /streptomycin /glutamine, 1% nonessential amino acids

(Gibco®). Medium was changed weekly until colonies

appeared. After 10-14 days colonies were picked using

5 mm cloning discs (Labcore®) soaked in 1X

trypine-EDTA (Life Technologies®) and cultured in 24

20   well tissue culture plates with CHO select medium.

After 1-2 weeks glycosylated leptin protein expression

was determined using a leptin EIA assay described

below. The best expressing clones (i.e., those which

demonstrated the most intense response using the EIA)

25   were expanded and frozen in cryogenic storage.

In some circumstances a more rapid protocol

was used to express analogs in CHO cells. In this case

electroporation was used to transfect cells and

individual clones were not isolated. Electroporation

30   experiments used 200 µg of pDSRα2 with the glycosylated

leptin protein insert as described above, and 200 µg of

herring sperm carrier DNA. The DNAs were phenol-

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chloroform extracted and ethanol precipitated, then
resuspended in 800 μl 1X HEBS along with 2 X 10^7 DHFR-
Chinese Hamster Ovary (CHO) cells grown in CHO D$^-$
medium. The cells and DNA were incubated at room

5   temperature for 10 minutes. Electroporation was
carried out at 290 volts, and 960 ufarads using a BIO
RAD Gene Pulser™ in 0.4 cm electroporation cuvettes.
Cells were then incubated for 10 minutes at room
temperature, washed with 10 ml CHO D- media, spun for

10   10 minutes at 1000 rpm in a Damon®/IEC Division IEC
HN-SII Centrifuge, then resuspended in 20 ml CHO D-
media and added to two 10 centimeter dishes. Cells
were grown for 2 days at 37°C, then split 1:4 into CHO
selection media and grown to ~70% confluency. Cells

15   were then split 1:2 into selection media plus 6 nM
methotrexate and grown at 37°C until clones were
visible (approximately 2 weeks). Pools were generated
from plates containing at least 4 colonies and were
grown in selection media with 6 nM methotrexate until

20   confluent (approximately 1 week). The pools were then
frozen in cryogenic storage.

Expression and purification of N48 T50 Leptin
(Single glycosylation site leptin protein).

CHO cells were transformed with DNA

25   expressing N48 T50 Leptin as described in above. Cells
were expanded in spinner culture in growth medium
(DMEM/F12 (1:1), 365 mg/l L-Glutamine, 1X MEM Non-
Essential Amino Acids, 5% FBS). Roller bottles with
breathable caps were then inoculated with 2e7

30   cells/bottle in 400 ml of growth medium and gassed for
10 seconds with 10% CO$_2$ in air. After 5 days, the
bottles were shifted to serum free production medium
(400 ml/bottle, DMEM/F12 (1:1), 365 mg/l (1X) L-
Glutamine, 1X MEM Non-Essential Amino Acids, 10μM

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CUSO4, 1.5g/l additional glucose). Serum-free
conditioned medium from three successive harvests were
collected (180 Liters) and filtered through a 0.45 μm
filter, concentrated about 30 fold, and diafiltered

5   into 1 mM CHAPS, 10 mM Tris, pH 7.5 using a tangential-
flow ultrafiltration system (Amicon®) with a 10,000
molecular weight cutoff membrane. The diafiltered
media was stored at -20°C.

The following steps were performed at 2 to

10   8°C. The DFM was applied to a Q-Sepharose Fast Flow
column (Pharmacia®, 8 cm x 14 cm) equilibrated in 10
mM Tris, pH 7.9 and washed with about two column
volumes of 10 mM Tris to elute all non-binding species.
N48 T50 leptin, which remains bound to the column, was

15   then eluted by a twelve column volume gradient from
10mM Tris, pH 7.9 to 200 mM NaCl, 10mM Tris, pH 7.9
collected into fractions. Fractions containing fully
glycosylated N48 T50 leptin, as determined by Western
blot analysis, were combined then diluted with one

20   volume of water to reduce the sodium chloride
concentration. The sample was then applied to a Bio-
Gel® HT column (Bio-Rad®, 10 cm x 7 cm) equilibrated
in 10mM Tris, pH 7.9 then washed with about four column
volumes of 10mM Tris, pH 7.9. Fractions of the non-

25   binding species were collected and those containing N48
T50 leptin, as determined by Western blot analysis,
were combined.

A one third volume of 3 M $(NH_4)_2SO_4$, 10mM
Tris, pH 7.9 was added to the N48 T50 leptin pool from

30   the Bio-Gel® HT column. The pool, now in 1 M
$(NH_4)_2SO_4$, was applied to a Source 15PHE column
(Pharmacia®, 10 cm x 1.6 cm) equilibrated in 1 M
$(NH_4)_2SO_4$, 10mM Tris, pH 7.9 then washed with about two

column volumes of 1 M (NH4)2SO4, 10 mM Tris, pH 7.9.

F3, which remains bound to the column, was then eluted

by a 40 column volume gradient from 1 M (NH4)2SO4, 10mM

Tris, pH 7.9 to 10mM Tris, pH 7.9 collected into

5   fractions. Fractions containing F3, as determined by

SDS-PAGE analysis, were combined.

Solid ammonium sulfate was added to the N48

T50 leptin pool from the Source 15PHE column to a final

concentration of about 2.5 M and incubated overnight.

10   The overnight precipitate was harvested by

centrifugation.

The harvested ammonium sulfate precipitate

was resolubilized in water, titrated to pH 4.5 with

acetic acid, applied to a Source 15S column

15   (Pharmacia®, 5.5 cm x 1.6 cm) equilibrated in 10mM

NaCH2COOH, pH 4.5, then washed with about two column

volumes 10mM NaCH2COOH, pH 4.5. N48 T50 leptin which

remains bound to the column was then eluted by a 72

column volume gradient from 50 mM NaCl, 10mM NaCH2COOH,

20   pH 4.5 to 150 mM NaCl, 10mM NaCH2COOH, pH 4.5 collected

into fractions. Fractions containing N48 T50 leptin,

as determined by SDS-PAGE analysis, were combined and

titrated to pH 7.5.

The N48 T50 leptin pool from the Source 15S

25   column was concentrated to about 1 mg/ml, diafiltered

into Dulbecco's PBS (Gibco®), then concentrated to

about 5 mg/ml using a stirred cell ultrafiltration

system (Amicon®) with a 10,000 molecular weight cutoff

membrane (Filtron®). The N48 T50 leptin was further

30   concentrated to 10 mg/ml using centrifugal

ultrafiltration (Centricon 10, Amicon®). The

concentrated N48 T50 leptin was filtered (0.22 µm) and

stored at 2 to 8°C.

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II.   Analytical Methods

        The following analytical methods were used
herein to characterize the present glycosylated leptin
5   proteins.

        A.   In vitro Assays

        1.   Receptor Binding Assay.   In this assay,
membrane-bound leptin receptor was used as a target to
measure the amount of binding by radioactively labeled
10   test glycosylated leptins.

        Chinese Hamster Ovary ("CHO") cells were
engineered to stably express a human leptin receptor by
transfecting them with human leptin receptor DNA (short
form; Tartaglia et al., Cell 83: 1271 et seq.(1995)
15   herein incorporated by reference in its entirety; the
entire article is herein incorporated by reference).
Leptin receptor expressing cells were grown and
collected by low speed centrifugation.   The pelleted
cells (approximately 50 mg wet weight) were resuspended
20   in 0.32 M sucrose/25 mM HEPES and homogenized in glass
homogenizing tubes using a Glas-col® motor.   The cell
membranes were washed two times by centrifugation
(48,000 x g), dispersion using a polytron
homogenizer(Tissue Tearor®), and resuspension in cold
25   binding buffer(MEM,Gibco BRL®/ 25 mM HEPES, Gibco
BRL®/0.1 % BSA/ 0.5 mg/ml Bacitracin®(Sigma®)/ 0.1
mg/mL STI, Boehringer Mannheim/ 0.1 mg/mL AEBSF,
Boehringer Mannheim).   After the second wash, the
membrane preparation was resuspended at a final
30   concentration of 2-3 mg wet weight/mL in cold binding
buffer.

        Competition binding was performed by
incubating 400 uL membrane solution, 50 uL of 2 nM
125I-Leptin (Amersham) and 50 uL sample or Leptin

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standard(10-6 M rHu-Leptin 1-146) for 2-3 hours at room
temperature in 12mm x 75mm tubes. Bound 125I-Leptin
was separated from unbound 125I-Leptin by filtration
through glass fiber filters and 3 washes with cold PBS
5   using a Brandel cell harvester. Bound radioactivity
was determined with a gamma counter. The affinity of
each analog for the leptin receptor was determined by
calculation of the midpoint of the cold displacement
curve (IC50) for each analog.
10   2. In vitro biological activity. In this
assay, in vitro biological activity was determined
using a chimeric leptin receptor, having an
extracellular domain of a leptin receptor, a
transmembrane and intracellular domains of an
15 erythropoietin receptor. Upon activation of the
intracellular erythropoietin receptor domain by binding
to the extracellular leptin domain, the cells exhibited
a biological activity of proliferation, measured by H^3-
thymidine uptake.
20   Interleukin-3 (IL-3) dependent 32D (clone3)
murine myeloid progenitor cells (Greenberger et al.,
PNAS-USA 80: 2931 (1983) herein incorporated by
reference) were grown in RPMI 1640 (Gibco®)
supplemented with 10% fetal bovine serum and 10 ng/mL
25 IL-3 (Biosource®). A chimeric leptin receptor-EPO
receptor (OBR-EPOR) was constructed by standard
techniques and subcloned into an expression vector
containing the transcriptional promoter of Moloney
murine sarcoma virus resulting in the vector
30 OBR-EPOR/pLJ. The chimeric receptor contained the
coding regions for the extracellular domain of a human
leptin receptor (amino acids 1-839; Tartaglia et al.,
Cell: 83: 1271 (1996) (herein incorporated by reference
in its entirety) and transmembrane and intracellular
35 domains of murine erythropoietin receptor (amino acids

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250-507; D'Andrea et al., 57: 277 (1989) herein
incorporated by reference in its entirety. The
chimeric receptor was then transfected into 32D cells
by electroporation. Transfected cells were initially
5   selected on G418 (750 ug/ml). Leptin responsive cells
were then selected in RPMI 1640 (Gibco®) supplemented
with 10% fetal bovine serum and 25 ng/ml Hu Leptin,
resulting in 32D-OBECA cells. 32D cells which were not
transfected with the chimeric receptor remained
10 unresponsive to leptin.

    32D-OBECA cells were grown in 1640 RPMI
medium (1x liquid, without L-Glutamine, Gibco®)
containing 10% fetal bovine serum (Hyclone
Laboratories®) and 1.0 ng/ml of recombinant murine
15 IL-3 (Biosource®) to a density of approximately
5.0E+05 cells/ml. Cells were collected by
centrifugation (approximately 270 X G), washed twice in
sterile 1X PBS (Gibco®) and then resuspended to
1.0E+05 cells/ml in a media consisting of 20% DMEM
20 Medium (DMEM+10%FBS) plus 80% assay medium (RPMI + 2%
FBS) plus 10ng/mL pan-specific anti-TGFβ neutralizing
antibody. An extended twelve point rmetHu-Leptin 1-146
standard curve was prepared using assay medium at a
range of approximately 0.1 to 200ng/ml. Test samples
25 were diluted in assay medium and typically run as
extended multiple point curves or at ranges falling
within the linear range of the standard curve. A
volume of 100μl of each sample was added to appropriate
wells of 96 well microtiter tissue culture plates.
30 Cells with sample or standard were grown at 10,000
cells per well (in 100μl) for approximately 48 hours at
5±1% CO_2 and 37±2°C high humidity incubator.
3H-Thymidine(0.5 μCi per well, Dupont®) was then added

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and the plates were incubated for an additional 18
hours and their DNA harvested onto preprinted glass
fiber filtermats (Pharmacia®) using a cell harvester
(Tomtek 96 Mach II®). Filters were dried in a

5   microwave oven, bagged in LBK® sample bags plus 10ml
of scintillation fluid (LKB®), then counted in a
Betaplate® scintillation counter (LKB®). Cell
response (in the form of average CPMs-background) was
plotted vs. mass (ng/well) of a rHu-Leptin 1-146

10   standard. The bioactivity of a sample of rmetHu-Leptin
or glycosylated leptin was determined from regression
analysis of the standard curve. Specific activity was
calculated by dividing the assayed biological activity
(ng/ml) by the concentration as determined by leptin

15   ELISA.
       B.   Characterization of glycosylated leptin
proteins
       1.   Enzyme Immuno Assay ("EIA").
       Polyclonal antibodies. Anti-rmetHu-Leptin

20   1-146 (SEQ. ID NO: 1 with a methionyl residue at the -1
position) polyclonal antibodies were raised in New
Zealand white rabbits by repeated subcutaneous
injections of rmetHu-Leptin 1-146 conjugated to keyhole
limpet hemocyanin (KLH), and mixed with the adjuvant

25   Titermax^{tm}, or with Freunds complete adjuvant (primary
injection)and Freunds incomplete adjuvant (subsequent
injections). The resulting rabbit sera were tested for
reactivity with rmetHu-Leptin 1-146, and sera from
those rabbits with the highest titer were pooled and

30   affinity purified over Actigel-ALD Superflow®
(Sterogene #2701-S-01) coupled to rmetHu-Leptin. An
aliquot of the purified polyclonal antibody was coupled
to horseradish peroxidase (HRP, Sigma® P-8415) to be

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used as a detecting antibody in the sandwich enzyme
immunoassay (EIA) or Western.

        Monoclonal antibodies. Anti-rmetHu-Leptin
1-146 monoclonal antibodies were developed from Lou
rats that were injected multiple times with KLH
conjugated rmetHu-Leptin 1-146, mixed with Freunds
complete adjuvant (primary injection) or with Freunds
incomplete adjuvant (subsequent injections). Rat sera
were tested for reactivity with rmetHu-Leptin 1-146,
and spleen cells from those with the highest titers
were fused to rat myeloma line Y3Ag 1.2.3 by standard
hybridoma techniques. The hybrid cells were plated in
96-well plates, allowed to form colonies, assayed for
anti-rmetHu-Leptin 1-146 activity, and single-cell
cloned. Monoclonal antibody from a rat hybridoma was
used as one component of the leptin sandwich EIA.

        Sandwich Assay. Microtiter plates (96-well
standard [Immulon®] or half-well [Costar®]) were
coated with 75µl or 30µl, respectively, of either
polyclonal (1.5 µg/ml) or monoclonal (2.0 µg/ml)
antibody in carbonate/bicarbonate buffer (NaHCO$_3$
0.029M, Na$_2$CO$_3$ 0.015M, pH 9.6). The plates were
blocked (1% bovine serum albumin [BSA], 5% sucrose) and
samples, diluted appropriately in 2% BSA in phosphate
buffered saline (PBS) were added, in duplicate, to the
wells. To each microtiter plate was also added, in
triplicate, a set of r-metHu-Leptin standards, or
glycosylated leptin standards, covering the range of 90
to 4580 pg/ml. The plates were incubated at 4°C for 18
hours, aspirated and washed three times with wash
buffer (Tris 50mM, NaCl 0.15M, EDTA 10mM, Tween® 20
0.05%, pH 7.35 [TEN]) and HRP-conjugated polyclonal
anti-rmetHu-Leptin 1-146 was added, ~70 ng/ml in 2% BSA

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in PBS with 0.05% Tween® 20. The plates were
incubated at room temperature for 3 hours, washed five
times with TEN and color developed with TMB substrate
(tetramethyl benzidine) according to the manufacturer's
5   instructions (Kirkegaard and Perry #50-76-00,
Gaithersburg, MD 20879). Absorbance was measured at
450nm in a microplate reader. Leptin concentrations
were calculated from a standard curve constructed for
each plate, after subtraction of background color.
10   Assay sensitivity was approximately 90 pg/ml; the
inter- and intra-assay variations, calculated from
controls included on each microplate, were 7.5% and
5.4%, respectively.

            2.   Carbohydrate Analysis by Western
15   Blotting.

            Generally, the larger the molecular weight of
a glycosylated leptin protein of the present invention,
the more heavily glycosylated. Thus the present
Western-blot type analysis was used to determine the
20   amount of carbohydrate present on the expressed
glycosylated leptin proteins.

            A volume of supernatant containing
approximately 400-600 pg of glycosylated leptin protein
from COS or CHO cells transfected with glycosylated
25   leptin protein cDNAs as described above was mixed with
SDS-PAGE 3X sample buffer (0.1875 M Tris-HCl pH 6.8, 6%
SDS, 30% glycerol, 15% 2-mercaptoethanol). The samples
were analyzed by 14% acrylamide Tris-Glycine
SDS-polyacrylamide gel electrophoresis (Novex®) and
30   transferred to 0.45 μm nitrocellulose
membrane(Novex®). The nitrocellulose membrane was
rinsed, blocked with TBST (Tris 20mM, NaCl 137mM,
Tween® 20 0.08%) containing 10% FBS and 2% BSA and

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incubated with HRP-conjugated polyclonal
anti-rmetHu-Leptin 1-146 as prepared above (140 ng/ml,
in TBST containing 5% FBS and 1% BSA) for 3-5 hours.
After washing with TBST, five times, five minutes each,
5   the membrane was developed with ECL reagent
(Amersham®), according to the manufacturer's
directions. The membrane was exposed to X-Omat® AR
film (Kodak®) for ten to sixty seconds, and developed
as for standard x-ray film. Specific protein bands
10   were visualized, and sizes estimated from their
positions relative to the molecular weight markers.
The larger the size, the more carbohydrate moiety
connected to the protein.

Treatment with N-glycanase. N-glycanase
15   treatment removes N-linked carbohydrate resulting in an
increase in mobility that is equal to that of
unglycosylated leptin. Treatment of glycosylated
leptin proteins with N-glycanase resulted in a
molecular weight similar to unglycosylated leptin.
20   This confirms that the increased size of glycosylated
leptin protein is due to N-linked carbohydrate.

Methods. COS cell conditioned medium
containing 400 pg of glycosylated leptin protein
(1-3ul) was mixed with 10 µl 0.5% SDS and each sample
25   was boiled for 3 minutes. Then 10.5µl of a 0.5M NaPO4
pH 8.6 + 7.5% nonidet P40 was added with 3 µl of 250
unit/ml N-glycanase (Genzyme®). Each sample was
incubated overnight at 37ºC and the reaction was
stopped by the addition of SDS-PAGE sample buffer and
30   subjected to SDS-PAGE Western analysis as described
above. These results indicate that the reduced
mobility on SDS PAGE observed is due to addition of
N-linked carbohydrate. The fact that numerous
glycosylated leptin proteins were identified indicates

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that there are multiple positions in leptin that can
support N-linked carbohydrate addition. Similar
results were obtained when the analogs were expressed
in 293 cells. Similarly, when multiple-glycosylation
5 site leptin proteins were treated with N-glycanase,
their mobility also changed to that of unglycosylated
leptin indicating that the mobility differences are due
to the presence of N-linked carbohydrate.

10   *   *   *

        While the invention has been described in
what is considered to be its preferred embodiments,
it is not to be limited to the disclosed
15 embodiments, but on the contrary, is intended to
cover various modifications and equivalents included
within the spirit and scope of the appended claims,
which scope is to be accorded the broadest
interpretation so as to encompass all such
20 modifications and equivalents.

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WHAT IS CLAIMED IS:

WE CLAIM:

1.   A glycosylated leptin protein having a
Stokes' radius greater than that of naturally occurring
glycosylated human leptin of SEQ. ID NO: 2 (rHu-Leptin
1-145).

2.   A glycosylated leptin protein having a
Stokes' radius equal to or greater than 30 Å, as
determined by gel filtration.

3.   A glycosylated leptin protein preparation
wherein each glycosylated leptin protein molecule in
said preparation has five or more sialic acid moieties.

4. A glycosylated leptin protein preparation
of claim 3 wherein each glycosylated leptin protein
molecule in said preparation has 8 to 20 sialic acid
residues.

5.   A glycosylated leptin protein comprising
human leptin of SEQ. ID NOs. 1 or 2 modified to contain
at least one additional site for glycosylation.

6.   A glycosylated leptin protein comprising
SEQ. ID NO: 1 having one or more sequence alterations
as a site of glycosylation selected from among (where
"T/S" denotes threonine or serine):

        (a) 01V->N 02P->X (where X is any amino acid
        except proline) 03I->T/S
            (b)   02P->N 03I 04Q->T/S
            (c)   23D->N 24I 25S->T/S
            (d)   47P->N 48I 49L->T/S
            (e)   48I->N 49L 50T/S
            (f)   69P->N 70S 71R->T/S
            (g)   92F->N 93S 94K->T/S
            (h) 101A->N 102S 103G->T/S

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(i) 102S->N 103G 104L->T/S

(j) 103G->N 104L 105E->T/S

7. A glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having a
glycosylation site located at a position selected from
among (with respect to the numbering of SEQ. ID NO: 1):
4, 8,23, 44, 47, 48, 69, 70, 92, 93, 97, 100, 101, 102,
103, 118 and 141.

8. A glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having two
glycosylation sites, said two sites selected from among
(with respect to the numbering of SEQ. ID NO: 1):
47 + 69;
48 + 69;
69 + 101;
69 + 102;
69 + 103;
69 + 118; and,
100 + 102.

9. A glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having three
glycosylation sites, said three sites selected from
among (with respect to the numbering of SEQ. ID NO: 1):
2 + 47 + 69
23 + 47 + 69;
47 + 69 + 100;
47 + 69 + 102;
48 + 69 + 118;
69 + 102 + 118; and,
69 + 103 + 118.

10. A glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having four

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glycosylation sites, said four sites selected from
among (with respect to the numbering of SEQ. ID NO: 1):

2 + 47 + 69 + 92;

2 + 47 + 69 + 102;

5   23 + 47 + 69 + 92;

23 + 47 + 69 + 102; and,

47 + 69 + 100 + 102.

11. A glycosylated leptin protein comprising
amino acids 1-146 of SEQ. ID NO: 1, having five

10   glycosylation sites, said five sites selected from
among (with respect to the numbering of SEQ. ID NO: 1):

2 + 23 + 47 + 69 + 92

2 + 47 + 69 + 92 + 102

23 + 47 + 69 + 92 + 102.

15

12. Glycosylated leptin 2,47,69 comprising
the amino acid sequence (SEQ. ID NO: 26):

20   1   VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT

51   LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPM

101 ASGLETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC

25

13. Glycosylated leptin 2,47,69,92
comprising the amino acid sequence (SEQ. ID NO: 28)

30   1   VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT

51   LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPM

101 ASGLETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC

35   14. Glycosylated leptin 2,47,69,102
comprising the amino acid sequence(SEQ. ID NO: 30):

1   VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT

40   51   LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPM

101 ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC

45   15. Glycosylated leptin 47,69,102 comprising
the amino acid sequence (SEQ. ID NO: 32)

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```
  1  VPIQKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL AFSKSCHLPW
101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

16.   Glycosylated leptin 2,47,69,92,102 comprising the amino acid sequence (SEQ. ID NO: 34):

```
  1  VNITKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPW
101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

17.   Glycosylated leptin 47,69,92,102 comprising the amino acid sequence (SEQ. ID NO: 36):

```
  1  VPIQKVQDDT KTLIKTIVTR INDISHTQSV SSKQKVTGLD FIPGLHNITT
 51  LSKMDQTLAV YQQILTSMNS TNVIQISNDL ENLRDLLHVL ANSTSCHLPW
101  ANGTETLDSL GGVLEASGYS TEVVALSRLQ GSLQDMLWQL DLSPGC
```

18.   A glycosylated leptin protein according to any of claims 1-6 having an N-terminal residue sequence selected from among:

a serine, arginine, proline or alanine residue at the -1 position,

a serine at the -1 position and a proline at the -2 position,

a serine-proline-serine sequence at the -1, -2, and -3 positions,

a serine at the -1 position and an arginine at the -2 position,

a serine at the -1 position, an arginine at the -2 position and a serine at the -3 position,

an arginine at the -1 position and a serine at the -2 position; and,

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an alanine at the -1 position and proline at
the -2 positions.

19. A nucleic acid encoding a glycosylated
leptin protein according to any of claims 1-6.

20. A nucleic acid encoding glycosylated
leptin 2,47,69 comprising the nucleic acid sequence
(SEQ. ID NO: 25):

```
  1  GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG
301  GCCAGTGGCC TGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTAAGCC CTGGGTGC
```

21. A nucleic acid encoding glycosylated
leptin 2,47,69,92 comprising the nucleic acid sequence
(SEQ. ID NO: 27):

```
  1  GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG
301  GCCAGTGGCC TGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

22. A nucleic acid encoding glycosylated leptin 2,47,69,102 comprising the nucleic acid sequence (SEQ. ID NO: 29):

```
  1 GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51 TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101 AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151 TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201 TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251 GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCCTGG
301 GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351 AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401 AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

23. A nucleic acid encoding glycosylated leptin 47,69,102 comprising the nucleic acid sequence (SEQ. ID NO: 31):

```
  1 GTGCCCATCC AAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51 TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101 AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151 TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201 TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251 GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGAGCTGCCA CTTGCCeTGG
301 GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351 AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401 AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

24. A nucleic acid encoding glycosylated leptin 2,47,69,92,102 comprising the nucleic acid sequence (SEQ. ID NO: 33):

```
  1 GTGAACATCA CAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51 TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101 AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151 TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
```

```
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG
301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

25.   A nucleic acid encoding glycosylated leptin 47,69,92,102 comprising the nucleic acid sequence (SEQ. ID NO: 35):

```
1  GTGCCCATCC AAAAAGTCCA AGATGACACC AAAACCCTCA TCAAGACAAT
 51  TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC
101  AGAAAGTCAC CGGTTTGGAC TTCATTCCTG GGCTCCACAA CATCACGACC
151  TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG
201  TATGAATTCC ACAAACGTGA TCCAAATATC CAACGACCTG GAGAACCTCC
251  GGGATCTTCT TCACGTGCTG GCCAACTCTA CCAGCTGCCA CTTGCCCTGG
301  GCCAATGGCA CGGAGACCTT GGACAGCCTG GGGGGTGTCC TGGAAGCTTC
351  AGGCTACTCC ACAGAGGTGG TGGCCCTGAG CAGGCTGCAG GGGTCTCTGC
401  AGGACATGCT GTGGCAGCTG GACCTCAGCC CTGGGTGC
```

26.   A vector containing a nucleic acid encoding a glycosylated leptin protein according to any of claims 19-25.

27.   A vector according to claim 26 which is an expression vector.

28.   A host cell containing a nucleic acid encoding a glycosylated leptin protein according to any of claims 1-6.

29.   A host cell of claim 28 selected from among prokaryotic and eukaryotic cells.

30.   A prokaryotic host cell of claim 29 which is a bacterial cell.

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31.   A eukaryotic host cell of claim 29 which is selected from among mammalian cells, yeast cells, and insect cells.

32.   A mammalian host cell according to claim 31 selected from among human cells, monkey cells, BHK cells and CHO cells.

33.   A method of preparing a protein according to any of claims 1-6 comprised of:

(a) culturing a cell containing a nucleic acid encoding said glycosylated leptin protein under suitable conditions for expression; and,

(b) obtaining said protein.

34.   A pharmaceutical composition for parenteral injection, intravenous injection, subcutaneous injection, intrathecal administration, nasal administration, pulmonary administration, and osmotic pump administration comprising a glycosylated leptin protein according to any of claims 1-18 in a pharmaceutically acceptable carrier.

35.   A method of treatment of a human for a condition selected from among obesity, diabetes, and high blood lipid content effects;

said method comprising administering an effective amount of a glycosylated human leptin according to any of claims 1-6.

36.   A method of treatment according to claim 35 wherein said effective amount of said glycosylated human leptin is administered by gene therapy.

37. A selective binding molecule which is selective for a glycosylated leptin protein according to any of claims 1-6.

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38.   A selective binding molecule of claim 37
which is selected from among a polyclonal antibody, a
monoclonal antibody, and a recombinant antibody.

39.   A method of manufacturing a glycosylated
5   leptin protein comprising:

(a) culturing, under suitable conditions for
expression, a host cell containing a DNA sequence
encoding, in the 5' to 3' direction (i) a signal
peptide, and (ii) a DNA encoding a glycosylated leptin
10   protein; and

(b) obtaining said glycosylated leptin
protein.

40.   A method of claim 39 wherein said signal
peptide is selected from among:

15   a) (SEQ. ID NO: 3) (native human leptin signal
peptide) MHWGTLCGFLWLWPYLFYVQA

(b) (SEQ. ID NO: 4) (modified human leptin
signal peptide) MHWGTLCGFLWLWPYLFYVSPS
20

(c) (SEQ. ID NO: 5)   (modified human leptin
signal peptide) MHWGTLCGFLWLWPYLFYVSP

(d) (SEQ. ID NO: 6) (modified human leptin
25   signal peptide) MHWGTLCGFLWLWPYLFYVSPA

(e) (SEQ. ID NO: 7) (modified human leptin
signal peptide) MHWGTLCGFLWLWPYLFYVSNS

30   (f)   (SEQ. ID NO: 8) (native human tPA signal
peptide) MDAMKRGLCCVLLLCGAVFVSPS

(g)   (SEQ. ID NO: 9) (native human tPA signal
peptide) MDAMKRGLCCVLLLCGAVFVSP
35

(h)   (SEQ. ID NO: 10) (modified tPA signal
peptide) MDAMKRGLCCVLLLCGAVFVSNS

(i)   (SEQ. ID NO: 11) (modified tPA signal
40   peptide) MDAMKRGLCCVLLLCGAVFVSPA

(j)   (SEQ. ID NO: 12) (Leptin/tPA signal
peptide) MHWGTLCCVLLLCGAVFVSPS

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(k)   (SEQ. ID NO: 13)(Leptin/tPA signal
peptide) MHWGTLCCVLLLCGAVFVSP

5   41. A method of claim 39 wherein said signal
peptide is selected from among the signal peptide for:
erythropoietin, Factor VIII, beta-interferon,
serum albumin, insulin, von Willebrand's factor, CD11α,
IgG, follistatin, intrinsic factor, G-CSF,
10 ceruloplasmin, and LAMP-1.
        42. An improved method of manufacturing a
glycosylated protein comprising:
        (a) culturing, under suitable conditions for
expression and glycosylation, a host cell containing a
15 DNA sequence encoding, in the 5' to 3' direction (i) a
signal peptide, and (ii) a DNA encoding a glycosylated
protein; and
        (b) obtaining said glycosylated protein;
wherein said improvement comprises use of a signal
20 peptide having a peptidase cleavage site optimized for
glycosylation efficiency.
        43. The improved method of claim 42 where
said peptidase cleavage site is selected from among
SPS, SP, SNS, and SPA.
25   44. A method of claim 39 or 42 which
optionally includes use of a prosequence.
        45. A nucleic acid encoding a signal peptide
having a non-naturally occurring peptidase cleavage
site.
30   46. A vector containing a nucleic acid
encoding a signal peptide having a non-naturally
occurring peptidase cleavage site.
        47. A vector according to claim 46 which is
an expression vector.

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48.   A host cell containing a nucleic acid
encoding a signal peptide having a non-naturally
occurring peptidase cleavage site.

49.   A host cell of claim 48 selected from
5   among prokaryotic and eukaryotic cells.

50.   A prokaryotic host cell of claim 49
which is a bacterial cell.

51.   A eukaryotic host cell of claim 49 which
is selected from among mammalian cells, yeast cells,
10   and insect cells.

52.   A mammalian host cell according to claim
51 selected from among human cells, monkey cells, BHK
cells and CHO cells.

FIG. 1

Legend:

— — Leptin (0.2 mg/ml, 1 mg/kg, 100ul)

—■— Leptin (2 mg/ml, 10 mg/kg, 100ul)

—◆— Glycosylated CHO Leptin (0.2 mg/ml, 1 mg/kg, 100ul)

—⊠— Glycosylated CHO Leptin (2 mg/ml, 10 mg/kg, 100ul)

—●— A4S Control (400ul, day 0)

—⊞— Leptin (5 mg/ml, 100 mg/kg, 400ul, day 0)

—▲— Glycosylated CHO Leptin (5 mg/ml, 100 mg/kg, 400ul, day 0)

FIG. 2

mock

W.T.

PWAS → SNAT (100a)

WAS → NAS (100b)

WAS → NAT (100c)

PWAS → ANAT (100d)

WAS → TAS (100e)

← N-Glycosylated
← O-Glycosylated
← Unglycosylated

FIG. 3

Serum Leptin Concentration (ng/mL) vs Time (h)

—△— Glycosylated Leptin
—○— r-metHuLeptin (from Study OBH.036)

FIG. 4

Serum Leptin Concentration (ng/mL) vs Time (h)

—△— Glycosylated Leptin
—○— r-metHuLeptin (from Study OBH.036)

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FIG. 5

- ●— Vehicle
- ✕— hLeptin 0.5 mg/kg/d
- △— GE-Leptin 0.5 mg/kg/d

FIG. 6

- ●— Vehicle
- ✕— hLeptin 0.5 mg/kg/d
- △— GE-Leptin 0.5 mg/kg/d

FIG. 7

N-glycosylation Sites
N47+N69+N102

FIG. 8

FIG. 9

N-glycosylation Sites N47+N69+N102

Signal Peptides

Clone Number

Leptin (SPS)   2   1

Leptin (SP)   2   1

Leptin/Tpa   2   1

Tpa/Leptin   2   1

Tpa   1

Leptin   1

Triple Glycosylated Leptin

Unglycosylated Leptin

FIG. 10

FIG. 11

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N15/16   C07K14/575   C12N15/63   C12N5/10   A61K38/22
        A61P3/04   A61P3/06   A61P5/48   C07K16/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 20933 A (SCHERING CORP) 12 June 1997 (1997-06-12) page 2, line 13-27; table 1 page 6, line 1-30 page 9, line 1-8 page 10, line 32 -page 11, line 12; examples I-V	1-33
Y	WO 95 05465 A (AMGEN INC) 23 February 1995 (1995-02-23) abstract page 5, line 10-25 page 13, line 28 -page 17, line 34 page 18, line 10-34; examples 4,5; tables 3-5	1-33

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 July 2000	31/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mateo Rosell, A.M.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	LE MAIRE M ET AL: "THE USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF SIZE AND MOLECULAR WEIGHT OF PROTEINS A CAUTION AND A LIST OF MEMBRANE PROTEINS SUITABLE AS STANDARDS" ANALYTICAL BIOCHEMISTRY, vol. 154, no. 2, 1986, pages 525-535, XP000925150 ISSN: 0003-2697 cited in the application the whole document	1,2
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 23115 A (RINELLA JOSEPH VINCENT JUNIOR ;LILLY CO ELI (US); BEALS JOHN MICHA) 14 May 1999 (1999-05-14) abstract page 2, line 22 -page 5, line 17 page 10, line 20 -page 11, line 15; examples 1-4 page 16, line 8 -page 18, line 9 page 18, line 10 -page 36, line 25	1-52
P,X	COUSIN PATRICE ET AL: "Influence of glycosylation on the clearance of recombinant human sex hormone-binding globulin from rabbit blood." JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, vol. 70, no. 4-6, September 1999 (1999-09), pages 115-121, XP000925370 ISSN: 0960-0760 the whole document	1-33
E	WO 00 09165 A (AMGEN INC) 24 February 2000 (2000-02-24) page 4, line 31-40 -page 5, line 1-9 page 11, line 39 -page 14, line 18; examples 3,4	1,5,6, 25-36
E	WO 00 21574 A (AMGEN INC) 20 April 2000 (2000-04-20) page 3, line 15-29 -page 5, line 1-20 page 11, line 12 -page 14, line 32; example 5	1,5,6, 26-36

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1,2

Present claims 1 and 2 relate to an extremely large number of possible leptins. In fact, the claims contain so many options, that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely glycosylated leptins having five or more sialic acid moieties.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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